LIPOPROTEIN PROFILING IN DOGS AND CATS

WITH GASTROINTESTINAL DISEASE OR HEPATIC DISEASE

A Dissertation

by

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ABSTRACT

Canine and feline hyperlipidemia is associated with various diseases and can cause clinically relevant complications, such as cutaneous xanthomas, hepatobiliary diseases (e.g., cholelithiasis, cholestasis, vacuolar hepatopathy, snf bilisty mucocele), pancreatitis, glomerular disease, lipemia retinalis, or peripheral neuropathy. However, dyslipidemia has not been extensively investigated in dogs and cats, maybe in part because dogs and cats are high-density lipoprotein (HDL) predominant species and the development of atherosclerosis is rare. This study aimed to evaluate the lipoprotein profile by continuous lipoprotein density profiling (CLPDP) method in dogs and cats with various gastrointestinal (GI) diseases, including exocrine pancreatic insufficiency (EPI), chronic enteropathy (e.g., idiopathic inflammatory bowel disease), and hepatic diseases. The CLPDP is a novel density gradient ultracentrifugation (DGU) technique, which uses a self-generating density gradient solution, bismuth sodium ethylenediaminetetraacetic acid (NaBiEDTA), and a fluorescent probe, N-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl-D-*erythro*-sphingosine (NBD C6-ceramide). We performed the partial validation assay of the CLPDP assay for use with canine serum samples. The intra- and inter- assay variability showed that the CLPDP was precise and reproducible for nominal low-density lipoprotein (LDL)/HDL and HDL, whereas the method was precise but not reproducible for TRL.

Our study showed that patients with GI diseases that lead to malassimilation (i.e., exocrine pancreatic insufficiency [EPI] and chronic enteropathies) most likely have decreased serum lipid concentrations consisting of LDL and high-density lipoproteins HDL compared to healthy control animals. Cats with hepatic lipidosis had divergent lipoprotein profiles compared to healthy control cats. The amounts of lipid within the density of 1.0374-1.0438 g/mL

distinguished healthy control cats from cats with HL with a sensitivity of 87% and a specificity of 90%. Dogs with liver disease (i.e., chronic hepatitis: CH or portosystemic shunt:PSS) had significantly different lipoprotein profiles compared to healthy control dogs. Dogs with PSS had significantly decreased levels of lipoprotein fractions compared to dogs with CH and healthy control dogs. These findings could potentially have diagnostic and prognostic implications in dogs and cats with GI disease. In addition, lipoprotein profiles may aid in the better understanding of the role of lipid metabolism in GI diseases and may even aid in the development of novel therapeutic approaches.

DEDICATION

I would like to dedicate this work to my father, Hirokazu Ban, and my mother, Yoko Ban. With their unconditional love and support, I am where I am today. Special thanks to my sister, Chinatsu Ban, who always encouraged me to pursue my goal and guided me to find joy in my life. My dedication, of course, goes to my beloved husband, Yasushi Minamoto, and our precious daughter, Akari. Yasushi has been very supportive and patient. Akari has brought a "light" in my soul.

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NOMENCLATURE

TRL	Triglyceride-rich lipoproteins
LDL	Low-density lipoprotein
HDL	High-density lipoprotein
DGU	Density gradient ultracentrifugation
EPI	Exocrine pancreatic insufficiency
IBD	Inflammatory bowel disease
CE	Chronic enteropathy
CH	Chronic hepatitis
PSS	Portosystemic shunt

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

I.1 Lipoprotein structure and classification

Lipoproteins are conglomerates of lipid and protein. The main function of lipoproteins is to transport water-immiscible lipids in the aqueous environment of the blood and across cell membranes.[1] Lipoproteins form a micelle-like structure as they have a hydrophilic exterior and a hydrophobic core.[2] Particularly, because triacylglycerol and cholesterol esters are hydrophobic, they have to be coated with amphipathic compounds: phospholipids, unesterified cholesterol, and proteins. The polar heads of the phospholipid and the free hydroxyl group of cholesterol are aligned towards the external polar environment in the blood stream. Triacylglycerol and cholesterol esters are stabilized in the core.

The density of the particle is influenced by the composition of lipids and proteins within the particle.[3] As density increases, the ratio of triacylglycerol to phospholipids and cholesterol decreases. In addition, there is a strong relationship between biological function and the density class. Thus, in humans, lipoproteins are commonly categorized into 5 classes based on their densities: chylomicrons, very low density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL). The term IDL is often used interchangeably with remnant particles. IDL has not been characterized in dogs and cats.[1] It is important to note that lipoproteins within a same class are not homogenous, rather they are metabolic continuums. There is a wide variety of particle sizes and chemical compositions within each class, and there is an overlap between them.[3]

I.2 Lipoprotein functions

As mentioned above, the density classes of lipoproteins are well associated with their biological functions. Chylomicrons are the largest particles and their main functions are to transport dietary triglycerides (TG) from the small intestine to adipose tissue and skeletal/cardiac muscle as well as to transport dietary cholesterol to the liver.[4] Fat-soluble vitamins, vitamin A, D, E, and K, are also incorporated into chylomicrons.[5] The chylomicron remnants are taken up by the liver and some vitamin components are stored in the lipocytes of the liver. Chylomicrons are the most abundant lipoproteins in the blood circulation for a few hours after the ingestion of a meal.[6] The other classes of lipoproteins are considered to be the endogenous lipoproteins.[1] VLDL are secreted from the liver in order to export endogenously synthesized triglycerides and deliver triglycerides to adipose and muscle tissues. VLDL lose triacylglycerol and apolipoproteins and become a smaller and denser particles, LDL, which mainly transport cholesterol to peripheral tissues and liver. HDL serve to remove cholesterol from peripheral cells and macrophages to the liver. This “reverse cholesterol transport” process is considered antiatherogenic in humans.

I.3 Lipoprotein metabolism

Lipoprotein metabolism is quite complex. Dominiczak et al. introduced three conceptual frameworks: the fuel transport pathway (metabolism of chylomicrons, VLDL, and remnant particles), the overflow pathway (metabolism of LDL), and the reverse cholesterol transport (metabolism of HDL).[4] This approach should aid in better understanding of the pathogenesis of lipid disorders and relevant therapeutic interventions.

I.3.1 Fuel transport pathway

The fuel transport pathway delivers TG to peripheral tissues by chylomicrons and VLDL. Dietary TG are incorporated into chylomicrons in the enterocytes and secreted into the lymph within intestinal lacteals. In peripheral tissues, TG within chylomicron are hydrolyzed into free fatty acids and glycerol by lipoprotein lipase (LPL) from the vascular endothelium. After losing TG, chylomicrons decrease in size and become chylomicron remnants. The remnants bind to the LDL receptor related protein (LRP) and LDL receptor and are internalized into hepatocytes. In contrast to the absorption of dietary TG, endogenously synthesized TG are secreted within VLDL particles from the liver. TG within VLDL are partially hydrolyzed by LPL. VLDL particles then decrease in size and become VLDL remnants. VLDL remnants are taken up by the liver via the LDL receptors. It is important to note that VLDL remnants exchange some TG with cholesterol ester within HDL in a process mediated by the cholesteryl ester transfer protein (CETP).

I.3.2 Overflow pathway

While VLDL remnants are taken up by the liver, further lipolysis occurs by hepatic triglyceride lipase (HTGL) from endothelium in the hepatic microvasculature. During this process, the remnants lose apolipoprotein C and E, decrease in size, and become LDL. More LDL particles are generated when the remnant supply from the fuel transport pathway is excessive. LDL is also taken up by binding to the LDL receptor in the liver. Their cellular uptake increases when LDL receptor expression increases because of a decrease in intracellular cholesterol concentration.

I.3.3 Reverse cholesterol transport and cholesterol recycling

HDL is assembled on the matrix of the apolipoprotein AI (apoAI).[4] ApoAI is secreted from the liver and intestine. It has been suggested that apoAI binds to phospholipid molecules as it leaves the cell and become pre-β1 HDL. Pre-β1 HDL acquires phospholipids and free cholesterol by interactions with peripheral cell membranes and become discoidal HDL. Discoidal HDL acquires more cholesterol by binding to the membrane ATP-binding cassette transporter A1 (ABCA1) that controls an efflux of free cholesterol from cells[4]. Cholesterol is subsequently esterified by lecithin-cholesterol acyltrasferase (LCAT). Discoidal HDL acquires phospholipids and free cholesterol from TRL via phospholipid transfer protein (PLTP). Accumulation of cholesterol esters leads to discoidal HDL becoming smaller spherical particles, called HDL3. Furthermore, by transferring cholesteryl esters to TRL in exchange for TG, HDL3 turns into larger particles, which are HDL2. Cholesterol esters within HDL2 are taken up by the scavenger receptor B1 in the liver. By losing cholesterol esters, HDL2 becomes lipid-poor apoAI, which is a precursor for HDL.

I.4 Clinical significance of lipoprotein profiling in human medicine

Epidemiological studies have shown that altered proportions of lipoproteins is associated with an increased risk of cardiovascular disease.[7] More specifically, elevated LDL-cholesterol and/or decreased HDL-cholesterol are associated with an increased risk of atherosclerotic cardiovascular disease (ASCVD).[7,8] The subendothelial retention of apolipoprotein B-containing lipoproteins has been thought to initiate the process of atherogenesis.[9] These retained lipoproteins promote a chronic and maladaptive macrophage-and T-cell-dominant inflammatory response, leading to a subsequent development of lesions.[10] Even though the etiology of ASCVD

is multifactorial, LDL-C has been widely used for cardiovascular risk assessment.[11] In addition, HDL is thought to have a protective effect on ASCVD because of its capacity to remove cholesterol and also its anti-inflammatory properties.[12] However, it has been controversial whether lowering LDL and/or raising HDL reduce the risk of ASCVD.[11,13,14] Several studies have shown that lipoprotein profiles as well as plasma cholesterol and triglyceride concentrations can be altered in human patients with inflammatory conditions.[15-17] In turn, chronic inflammatory disorders, such as rheumatoid arthritis, systemic lupus erythematosus (SLE), and inflammatory bowel disease (IBD) are associated with an increased risk of atherosclerotic cardiovascular disease in humans.[17]

I.5 Clinical significance of lipoprotein profiling in veterinary medicine

Hyperlipidemia is defined as an increased concentration of plasma triglycerides and/or cholesterol, and can due to excessive production and/or delayed degradation of lipoprotein. Hyperlipidemia is generally associated with various diseases and can cause clinically relevant complications, such as cutaneous xanthomas,[18] liver disease,[19] cholelithiasis,[20] pancreatitis,[21] glomerular disease,[22] lipemia retinalis, or peripheral neuropathy.[23] Moreover, altered lipid profiles have been reported in dogs with obesity,[24,25] hyperadrenocorticism,[26] dominance aggression,[27] lymphoma,[28] and some infectious diseases.[29,30] The causes of hyperlipidemia in dogs are either primary or secondary to other diseases. Secondary hyperlipidemia is the most common form and several diseases, such as hypothyroidism, diabetes mellitus, or pancreatitis, have been associated with the development of secondary hyperlipidemia. Primary hyperlipidemia is also often associated with certain breeds, such as Miniature Schnauzers or Shetland Sheepdogs.[21,31-33] On the other hand, idiopathic hyperchylomicronemia[18,34] and idiopathic hypercholesterolemia have been

reported in cats. Ginzinger et al. showed that cats with autosomal recessive defect in lipoprotein lipase activity had primary hyperlipidemia.[34] The clinical usefulness of measurement of the plasma cholesterol concentration in dogs and cats is minimal because it is not associated with development of complications as is the case in humans. On the other hand, hypertriglyceridemia has been reported to be a risk factor for pancreatitis in Miniature Schnauzer,[35] hepatobiliary disease (e.g., cholelithiasis, cholestasis, vacuolar hepatopathy, snf bilisty mucocele),[36] atherosclerosis,[37] and ocular disease, [38,39] seizures and other neurologic signs.[40,41]

I.6 Assessment of biological functions of lipoproteins

To assess lipoproteins qualitatively or quantitatively, the lipid contents of each lipoprotein subclass (e.g., LDL-Cholesterol) and the concentrations of apolipoproteins (apoB48/B100/AI) are often evaluated. On the other hand, to assess the biological function of lipoproteins, several characteristics of lipoprotein properties can be analyzed. For example, measurement of the lecithin:cholesterol acyltransferase (LCAT) activity, HDL cholesterol efflux capacity, and HDL antioxidant capacity represent the biological function of lipoproteins. It is also important to note that in human medical settings, plasma/serum triglyceride and cholesterol concentrations as well as LDL-cholesterol (LDL-C) and HLD-cholesterol (HDL-C) are commonly measured.

I.7 Methodology for lipoprotein profiling

As mentioned above, in human medicine, the standard method to assess LDL and HDL levels in clinical practice is to quantify the cholesterol content of LDL and HDL particles by the Friedewald equation. This is an effective diagnostic test because delayed LDL clearance results in increased plasma LDL-C as well as increasing the modifications of LDL particles[42] that make

them atherogenic.[43-46] However, lipoproteins are highly heterogeneous particles, and cholesterol concentrations within the lipoprotein particles do not necessarily reflect their biological properties.[47] In fact, although it has been well recognized that increased plasma LDL-cholesterol concentrations are associated with an increased risk for cardiovascular disease (CVD), a relatively high proportion of patients with CVD have plasma LDL-C concentrations within the reference interval.[48] Other methodologies have been introduced to assess the lipoprotein profile, including density gradient ultracentrifugation DGU), proton nuclear magnetic resonance (NMR), vertical auto profile (VAP), high performance liquid chromatography (HPLC).

I.7.1 Friedewald method

The standard method for assessing LDL level in clinical patients is to calculate the estimated cholesterol content in LDL particles by the Friedewald equation (LDL-C= total cholesterol – HDL-C – triglycerides/5) after precipitation of apoB-containing particles.[49] In fact, even though epidemiological studies have shown a consistent inverse association between HDL-C and cardio vascular disease (Boekholdt et al., 2013), clinical trials have shown that raising HDL-C pharmacologically does not reduce the incidence of CAD (Schaefer, 2013; Hafiane and Genest, 2015b). This finding might suggest that HDL-C is not directly responsible for the development of CAD, but rather something correlated with it. In other words, cholesterol concentrations within HDL might not represent antiatherogenic properties of HDL. Therefore, it would be more meaningful to evaluate overall lipoprotein distributions rather than cholesterol concentrations within lipoprotein particles (Larner et al., 2011; Hafiane and Genest, 2015a).

I.7.2 Density gradient ultracentrifugation

Density gradient ultracentrifugation (DGU), including rate zonal ultracentrifugation and isopycnic ultracentrifugation, has been the gold standard to separate and identify lipoproteins based on their density (Chapman et al., 1981). Typical DGU protocols are often very time-consuming because they require sequential centrifugation steps, and cannot capture the continuous density distribution of lipoproteins. In addition, lipid content (i.e., cholesterol and triglyceride concentrations) is usually measured by enzymatic assays following the ultracentrifugation isolation steps.

I.7.3 Nuclear magnetic resonance spectroscopy

Proton nuclear magnetic resonance (NMR) spectroscopy can measure lipoprotein particle numbers and sizes instead of the cholesterol content of LDL and HDL.[50] Each lipoprotein subclass has a different size and emits distinctive NMR signal and the signal amplitude measured for each subclass is directly proportional to the number of subclass particles emitting the signal. Lipoprotein sizes and particle numbers provide additional clinically useful information about the lipid profile for a specific individual because patients with same LDL-C concentrations can have significantly different numbers and sizes of LDL particles and a different risk for cardiovascular diseases.[50] Jeyarajah et al. showed a strong correlation between chemically measured and NMR measured triglycerides ($r = 0.978$) and HDL-C ($r = 0.959$) concentrations in human plasma samples.[50] Mora et al. showed that cardiovascular disease risk prediction evaluated by lipoprotein size and numbers measured by NMR was comparable but not superior to that of standard measurements of lipids and apolipoproteins in the prospective study of healthy

women.[51] Further studies are warranted to investigate the clinical utility of NMR spectroscopy for the evaluation of lipoproteins.

I.7.4 Vertical auto profile

The vertical auto profile (VAP) is an inverted rate zonal, single vertical spin, density gradient ultracentrifugation technique that simultaneously measures cholesterol concentrations of five lipoproteins classes and their subclasses.[52] The VAP method uses a vertical rotor and separates lipoproteins across the shorter horizontal axis of the centrifuge tube, which allows separating all lipoproteins in less than 1 hour. After centrifugation, the contents are drained from the bottom of the tube by puncturing it with a needle, and are slowly and continuously mixed with a flowing enzymatic reagent. As the mixture flows through the tube, the absorbance corresponding to the intensity of the red color is monitored with a spectrophotometer at 505 nm.[52,53]

I.7.5 High performance liquid chromatography

High performance liquid chromatography (HPLC) has been widely used for the separation and analysis of many substances. There are three types of HPLC columns for biological samples: reversed-phase (RPHPLC), ion-exchange (HPIEC), gel-permeation (HPGPC) or size-exclusion (HPSEC) columns.[54] HPGPC is recommended for separation of serum lipoproteins because they are large spherical particles with lipid core and hydrophilic outer layer, and the size of different classes of lipoprotein sizes differ remarkably, which allows separation through an HPGPC column.[54] The amount of each lipoprotein class can be quantified by calculating the area under the curve. In contrast, RPHPLC and HPGPC are usually used for analysis of apolipoproteins.[54] The advantages of HPLC for serum lipoprotein analysis include

shorter processing and analysis time, high resolution and reproducibility, and a small sample volume.[54]

I.7.6 Gel electrophoresis

Several types of gel electrophoresis have been used to determine lipoprotein profiles, but it is important to note that different types of gel electrophoresis serve a different purpose for lipoprotein analysis. Agarose gel electrophoresis is used to classify lipoproteins by the nomenclature beta, pre-beta, and alpha based on the mobility of LDL, VLDL, and HDL, respectively.[55] Non-denaturing polyacrylamide gradient gel electrophoresis (ND-PAGGE) has been used to separate lipoprotein subclasses based on their diameter size. Two-dimensional gradient gel electrophoresis (2D-PAGGE) uses the surface charge and particle size of lipoproteins to separate lipoprotein subclasses.

I.7.7 Measurements of apolipoproteins

Apolipoproteins are proteins within lipoprotein particles. The main functions of apolipoproteins include providing structural components for assembly of lipoprotein particles, and regulating lipoprotein metabolism.[4,5] ApolipoproteinB100 (apoB100) is synthesized in hepatocytes and its main function is to serve as a structural component of VLDL and LDL as well as serving as a ligand for LDL-receptors for uptake by the liver. One molecule of apoB resides per lipoprotein particle. Thus, it is a marker of the number of lipoprotein particles.[4] Apolipoprotein B48 (apoB48) is a structural component of chylomicrons. ApoB48 and apoB100 are transcribed from a single gene. Since one molecule of apoB48 is present in each chylomicron particle, it serves as a marker of the number of chylomicrons and their remnants. ApolipoproteinAI (apoAI) constitutes 70% of HDL apolipoproteins. It activates LCAT and

possesses anti-inflammatory and antioxidant properties. Serum concentration of apoAI is the most important marker of the activity of reverse cholesterol transport. Measurements of the concentrations of apoAI and apoB are carried out by immunological methods using antibodies raised against specific epitopes. Current techniques include enzyme-linked immunosorbent assays (ELISA), immunonephelometry, and imunoturbidimetry.[4]

I.7.8 Continuous lipoprotein density profiling

As mentioned above, typical DGU techniques are often very time-consuming because they require sequential centrifugation steps. Continuous lipoprotein density profiling (CLPDP) is a novel DGU technique, which uses a self-generating density gradient solution, bismuth sodium ethylenediaminetetraacetic acid (NaBiEDTA), and a fluorescent probe, NBD C6-ceramide.[56] The advantages of this technique are that it requires only one ultracentrifugation step, lasting 6 hours, and allows the investigator to visualize a continuous distribution of lipoproteins (lipoprotein profile) and to estimate the concentrations of lipoprotein subfractions by calculating the area under the curve. Henriquez et al. reported that CLPDP was able to distinguish between a control group and a cardiovascular disease group without assessing the traditional risk factors, elevated levels of LDL-C and/or decreased levels of HDL-C.[2] Xenoulis et al. investigated the usefulness of the CLPDP in canine serum and reported lipoprotein profiles in healthy dogs of various breeds, healthy miniature schnauzers, and miniature schnauzers with hyperlipidemia.[56]

I.8 Lipodomic changes in human IBD patients and in canine and feline patients with chronic gastrointestinal disease

Inflammatory bowel disease (IBD) is an umbrella term used to describe disorders characterized by persistent or recurrent GI signs and histologic evidence of intestinal

inflammation in the absence of an identifiable trigger or infection.[57] There are two major forms of IBD in humans: ulcerative colitis (UC) and Crohn's disease (CD).[58] The difference between UC and CD is mostly based on the distribution of affected sites. UC is a severe ulcerating inflammatory disease that is limited to the colon and rectum affecting the mucosa and submucosa. In contrast, CD may involve any area of the GI tract, frequently involving the ileum, and is typically transmural.[59] IBD in humans is clinically and histologically different from IBD in cats and dogs even though the etiology might be similar.[57] Intestinal inflammation is thought to be caused by a complex interaction between genetic, environmental and microbial factors, and the immune system.[60] However, the etiology of IBD remains largely unknown. [57] It has been previously recognized that IBD is associated with dyslipidemia. Lipid profile in a clinical setting in human medicine mostly include total plasma cholesterol, low-density lipoproteins cholesterol (LDL-C), and high-density lipoproteins cholesterol (HDL-C). IBD is associated with lower levels of total cholesterol and LDL-C, and either increased or unchanged levels of triglyceride and HDL-C.[15,61] Metabolomic studies have reported a number of disrupted metabolic pathways in IBD patients.[62] Using a lipidomics approach, it has been revealed that lipid metabolism, mainly sphingomyelin (SM) and ceramide metabolism, are significantly altered in an experimental IBD mouse model.[63] Moreover, lipid metabolism and its signaling are suggested to play important roles in the pathogenesis of IBD.[64]

I.8.1 Phospholipids

I.8.1.1 Glycerophospholipid

Three classes of membrane lipids, glycerolipids, sphingolipids and steroids, are widely distributed throughout the body.[5] Of these, glycerolipids are quantitatively by far the most

important group of membrane lipids. They can be divided into two main groups: those containing phosphorus (phosphoglycerides) and those without phosphorus but containing a sugar moiety (glycosylglycerides), both of which are based on a glycerol backbone.[5] Phosphoglycerides dominate in higher animals, whereas glycosylglycerides are more important in plants. Recently, Fan et al. investigated the relationship between the individual molecular species of lipids and human IBD.[64] They analyzed lipid profiles consisting of 333 lipid species in plasma of IBD patients (UC: n=16 and CD: n=24) and healthy volunteers (n=84). Plasma total cholesterol and LDL-C concentrations of patients with CD were lower than the ones in patients with UC. The lipidomic profiles were profoundly different in patients with CD but not with UC when compared with healthy controls. Most importantly, a number of ether lipids (i.e., alkylphospholipids and alkenylphospholipids (a.k.a., plasmalogens)) was negatively associated with CD, but not with UC. It has been known that the vinyl ether linkage of plasmalogens is particularly susceptible to oxidation by reactive oxygen species (ROS).[65] Therefore, plasmalogens serve as antioxidants and protect other lipids in lipoproteins and membranes against oxidation. The decrease of plasmalogens may lead to impaired antioxidant defense, thus contributing to the pathology of IBD.[64] The differences in lipidomic profiles between patients with CD and those with UC may be due to the fact that CD affects larger areas of the intestinal tract. Thus, lipidomic profiling might serve as a potential diagnostic modality for evaluating disease severity. Lower levels of plasmalogens in patients with CD may be due to enhanced oxidative decomposition of plasmalogens because an increase in oxidative stress is associated with IBD. In contrast, the fact that there is no significant decrease in plasmalogens in UC patients may be due to less intestinal area being affected in patients with this condition.

I.8.1.2 Sphingolipids

Sphingolipids contain a sphingosine or ceramide rather than a glycerol as a backbone.[5,66] Sphingolipids are widespread in membranes but are particularly abundant in nervous tissue. In addition, they are concentrated in the outer layer of the plasma membrane. Recent studies have shown that sphingolipids play a role in modulating various cellular events including proliferation, differentiation, apoptosis, and inflammation.[66] Two sphingolipid classes, the ceramide precursor dihydroceramide and the ceramide metabolite trihexosylceramide as well as 11 sphingolipid species, including 4 sphingomyelin species in plasma were found to be negatively associated with human IBD, suggesting a possible dysregulation of sphingolipid metabolism in human IBD patients.[64] Sewell et al. quantified the concentrations of individual ceramide and dihydroceramide species, sphingoid bases, and total ceramide content in macrophages from healthy subjects (n=7) and patients with CD (n=8).[67] Sphingolipid composition of macrophages is altered after stimulation with E. coli but did not differ between macrophages isolated from HC or CD patients.

I.8.2 Fatty acids and sterols

It has been suggested that fatty acid metabolism is involved in the immune response and inflammatory processes in human IBD patients. Esteve-Comas et al. described an abnormal polyunsaturated fatty acids (PUFA) pattern, consisting of increased levels of α-linoleic acid (C18:3n3) and docosahexaenoic acids (DHA, C22;6n3) and decreased level of dihommo-γ-linoleic (C20:3n6), in patients with active IBD.[68] In their study, even in patients with severe disease, plasma concentrations of the n3-PUFA remained higher than those in healthy controls.[68] These findings suggest that the synthesis of PUFA might be enhanced in patients with active

IBD and that the utilization of n6 PUFA might be increased due to increased demands for biosynthesis of eicosanoids. In order to examine this hypothesis, the same investigators later compared plasma fatty acid concentrations between patients with inactive IBD (n=24, UC without colectomy; n=15, UC with colectomy; n=27, CD) and healthy controls (n=107). Plasma concentrations and percentage of DHA was significantly higher in patients with inactive UC and CD than in controls. Arachidonic acid (C20:4n6), the main fatty acid of the n6 series, showed the same tendency, but the difference did not reach statistical significance. These results indicate that in patients with active IBD, the biosynthesis of PUFA (both n3 and n6) as well as the utilization of n6 PUFA for production of eicosanoids are enhanced, while in inactive IBD cases, concentrations of n6-PUFAs are even higher compared to healthy controls because the utilization of n6-PUFA is decreased due to a decrease in disease activitity. These investigators also pointed out that these results brought up a concerning potential negative effect of the use of long chain n3-PUFA in treatment for either acute attacks of IBD or for maintenance treatment of the disease.

In veterinary medicine, there are several studies that evaluated lipoprotein profiles using traditional methods, including the measurement of plasma total cholesterol, LDL-C, HDL-C. However, limited published reports regarding lipidomics are available. Honneffer et al. evaluated both sterol and fatty acid concentrations in healthy control dogs and dogs with EPI undergoing enzyme supplementation.[69] Their study showed that concentrations of all fecal fatty acids were significantly increased in dogs with EPI. The fatty acids evaluated in this study included essential fatty acids, such as linoleic acid and α-linoleic acid, and other unsaturated fatty acids, such as exhisonic acid, erucic acid, gondoic acid, and oleic acid. Among sterols, cholesterol concentrations did not significantly differ between both groups, while coprostanol was decreased

along with several phytosterols (i.e., sitosterol, campesterol, fucosterol, sitostanol, and stigmasterol).[69] This study suggested that fat assimilation does not normalize completely with enzyme supplementation even when clinical signs of EPI are well-managed.

The effect of cholestyramine on lipid metabolism was studied by measuring fecal fatty acids and sterol concentrations in healthy dogs before and after administration of cholestyramine.[70] Cholestyramine forms insoluble complexes with bile acids, preventing intestinal reabsorption of bile acids and thus enhancing their fecal elimination. As expected, the concentrations of all measured sterols were significantly decreased after administration of cholestyramine, including phytosterols (i.e., sitostanol, campesterol, sitosterol, stigmasterol, fucosterol) and zoosterols (i.e., cholesterol, coprostanol, and cholestanol). Several fecal fatty acid concentrations increased after administration of cholestyramine (i.e., oleic acid, stearic acid, and palmitic acid), while only linolenic acid decreased significantly. This result suggests that cholestyramine may have a significant effect on intra-luminal homeostasis of sterols and assimilation of fatty acids.

I.8.3 Total cholesterol, LDL-C, and HDL-C

In humans, IBD patients exhibit lower plasma concentrations of LDL-C and total cholesterol compared to healthy subjects. These differences are more profound in CD patients than UC patients. In addition, HDL-C and TG concentrations do not differ between IBD patients and healthy subjects.[61] These findings remain consistent independently of disease activity and are also observed post-operatively.[71] Even though the pathogenetic mechanism causing these alternations have not been fully elucidated, lipid metabolism has emerged as an important mediator for the development of inflammatory diseases, including IBD. Several pathways of the

mechanism of alternations of lipid metabolism have been revealed. During the early stages of inflammation, serum concentrations of VLDL and TG are increased. It has been suggested that the effects of infection and inflammation on TG metabolism are similar in all species, while changes in cholesterol metabolism differ between rodents and primates.[72] In rodents, serum total cholesterol concentrations and hepatic cholesterol synthesis are increased; on the other hand, humans and nonhuman primates have either no changes or show a decrease in serum cholesterol and low-density lipoproteins (LDL). Consequently, inflammatory conditions lead to alternations in the composition and function of lipoproteins, including changes in sphingolipid concentrations, decreased reverse cholesterol transport, and increased oxidation of lipids. As a result, high-density lipoproteins (HDL) concentrations are decreased in these patients.[72]

I.9 Hypotheses and specific objectives

I.9.1 Hypotheses

The hypotheses of this study are 1) that the CLPDP would be a useful tool for the assessment of lipoprotein profiles in dogs and cats and 2) that dogs and cats with gastrointestinal disease or chronic enteropathy and hepatic diseases have altered lipoprotein profiles measured by CLPDP when compared to healthy controls.

I.9.2 Specific objectives

The objectives to prove or disapprove these hypotheses were: 1) to perform partial inter- and intra-assay validation of the CLPDP with use of canine serum samples; 2) to evaluate the effects of freeze-thaw cycles of canine serum samples on the results of CLPDP; 3) to perform short- and long- term stability studies on canine serum samples kept under different storage

conditions; and 4) to evaluate lipoprotein profiles in dogs and cats with various gastrointestinal diseases.

CHAPTER II

ANALYTICAL ASSESSMENT OF CONTINUOUS DENSITY GRADIENT ULTRACENTRIFUGATION PROFILING METHOD FOR USE WITH CANINE SERUM

II.1 Introduction

In humans, the standard method for assessing LDL level in clinical practice is to quantify plasma LDL-C concentration by calculating estimated cholesterol contents in LDL particles by the Friedewald equation (LDL-C= total cholesterol – HDL-C – triglycerides/5).[49] This is an effective diagnostic test because delayed LDL clearance results in increased plasma LDL-C as well as increasing the modifications of LDL particles[42] that make them atherogenic.[43-46] However, lipoproteins are highly heterogeneous particles, and cholesterol concentrations within the lipoprotein particles do not necessarily reflect their biological properties.[73] Therefore, it could be more meaningful to evaluate entire lipoprotein distributions rather than cholesterol concentrations within lipoprotein particles.[73,74]

Density gradient ultracentrifugation (DGU), including rate zonal ultracentrifugation and isopycnic ultracentrifugation, has been the gold standard for separating and identifying lipoproteins based on their density.[75] Typical DGU techniques are often very time-consuming because they require sequential centrifugation steps. Continuous lipoprotein density profiling (CLPDP) is a novel DGU technique, which uses a self-generating density gradient solution, bismuth sodium ethylenediaminetetraacetic acid (NaBiEDTA), and a fluorescent probe, NBD C6-ceramide (N-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl-D-*erythro*-sphingosine).[56,74] The advantages of this technique are that it requires only one ultracentrifugation, lasting 6 hours, and allows visualization of a continuous distribution of

lipoproteins (lipoprotein profile) and also quantification of lipoprotein subfractions by calculating the area under the curve (AUC). The CLPDP was initially developed to analyze human lipoprotein profiles but was used in other species as well.[56,76,77] A recent study used this technique to analyze canine lipoprotein profiles in healthy dogs.[56] However, this technique has not been analytically validated in canine serum samples, and the stability of lipoproteins for use with canine serum and the effect of freeze-thaw cycles with different storage conditions on lipoprotein profiles in canine serum have not been investigated.

It is important to note that HDL is the predominant lipoprotein in dogs, whereas in humans, LDL predominates.[1] In addition, there is overlap in the density distributions of canine LDL and HDL particles, making it impossible to separate LDL and HDL completely by density centrifugation alone.[78,79] Thus, the density distribution of human lipoproteins cannot be simply applied to canine lipoprotein classification. The diameters of LDL and HDL lipoproteins in humans and dogs have been reported previously.[80] One way to evaluate the application of the CLPDP in canine samples would be to measure diameters of canine lipoproteins in two ranges, corresponding to human LDL and HDL. Observing the distribution of lipoprotein sizes in lipoprotein profiling by the CLPDP would help us better understand how this technique separates canine lipoproteins. The aims of this study were 1) to partially validate the CLPDP technique for lipoprotein profiling in canine serum, 2) to assess the stability of lipoproteins in canine serum samples under different storage conditions, 3) to assess the freeze-thaw cycle effects on lipoprotein profiling in canine serum, and 4) to investigate the shapes and sizes of both canine and feline lipoprotein particles using electron microscopy.

II.2 Materials and methods

II.2.1 Nomenclature

For the purpose of assay validation, stability assessment, and electron microscopic analysis, fractional lipoprotein intensities of TRL (1.0102-1.0190 g/mL), nominal LDL/HDL, (1.0191-1.0629 g/mL), and nominal HDL (1.0632-1.1781 g/mL) were determined by measuring the area under the curve (AUC) of the each density range. AUC% for the 3 density regions were calculated by normalizing by a total AUC (AUC for each density range/a total AUC=AUC%).

II.2.2 Assay repeatability and reproducibility

Partial validation test was performed for assay repeatability and reproducibility. Surplus serum samples submitted to the Gastrointestinal Laboratory (GI Lab) at Texas A&M University were pooled and aliquoted for determination of both intra-(repeatability)and inter-(reproducibility) assay validation. The intra-assay validation was performed at the same day. The rest of aliquots were kept at -80°C and inter-assay validation was performed within 3 months. Both AUC and %AUC were calculated for TRL, nominal LDL/HDL, and nominal HDL. Repeatability was determined by calculating the intra-assay coefficient of variation (%CV) of each density region (TRL, LDL/HDL, and HDL) from one serum sample assayed 10 times within the same analysis. Reproducibility was evaluated by calculating the inter-assay %CV of each density region (TRL, LDL/HDL, and HDL) from one serum sample in 10 different runs, each on a different day.

II.2.3 Effects of freeze-thaw cycle

Blood samples were obtained from 4 healthy dogs. Food was withheld for at least 12 hours prior to blood collection. Written informed consent was obtained from all owners and the

study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Texas A&M University (IACUC2014-0109CA). Blood samples were collected in plain glass tubes and left to clot for at least 40 minutes at room temperature. The samples were then centrifuged for 15 minutes at 1,600 × g and 20°C. Immediately after the centrifugation, lipoprotein profiling was performed as a baseline. The rest of the serum samples were aliquoted and stored at -80°C until analysis. The samples were thawed and refrozen up to 3 times, and analyzed at each point in the freeze-thaw cycle (1, 2, and 3) within 5 days after the blood collection. The %CVs of each density region (TRL, LDL/HDL, and HDL) were calculated between the baseline and each freeze-thaw time point.

II.2.4 Stability of lipoproteins in canine serum samples

Blood samples were obtained from 3 healthy dogs. The protocol for the blood draw was as mentioned above, and the same AUP (IACUC2014-0109CA) was used. Written informed consent was obtained from all owners of enrolled dogs. Lipoprotein profiling was performed at the same day of the blood collection as a baseline. The rest of the serum samples were aliquoted and stored at 4°C, -20°C, and -80°C until analysis. The serum samples stored at 4°C and -20 °C were analyzed after 3 days, 1 week, and 1 month. The serum samples stored at -80°C were analyzed after 3 days, 1 week, and 1, 3, 5, 6, 8, 9, 10, 11, and 12 months. The %CVs of each density region (TRL, LDL/DHL, and HDL) were calculated between the baseline and each time point under each storage condition.

II.2.5 Continuous lipoprotein density profiling (CLPDP)

The CLPDP was carried out as described previously with some modifications.[56,81] Briefly, 1280 µL of 0.18 M NaBiEDTA density solution (Tokyo Chemical Industry Co., Ltd.,

Tokyo, Japan) was mixed with 10 µL of serum and 10 µL of NBD C6-ceramide (Cayman Chemical Company, Ann Arbor, MI). Then 1150 µL of the mixture was transferred into a polycarbonate centrifuge tube (Beckman Coulter, Brea, CA). The sample tubes were centrifuged at 867,747 × g and 4^{o}C for 6 hours in Optima MAX-LP ultracentrifuge (Beckman Coulter, Brea, CA) with a fixed angle MLA-130 rotor (Beckman Coulter). After centrifugation, the tubes were immediately imaged by a fluorescence imaging system consisting of a digital camera (Quantifire XI, Optronics, Muskogee, OK) and a metal halide continuous light source (Dolan-Jenner Industries, Boxborough, MA). Following ultracentrifugation, the image of each tube was converted to a density profile using a software program, OriginPro7.5 (OriginLab, Northampton, MA). The tube coordinate (mm) on the X-axis of the lipoprotein profile corresponded to an actual centrifuge tube coordinate (mm). Zero mm indicated the top of the tube, and 33 mm indicated the bottom of the tube. The average intensity of fluorescence was plotted on the Y-axis to produce a lipoprotein profile. The less dense particles, such as triglyceride-rich lipoproteins (TRL), migrated near the top of the tube, whereas more dense particles, such as HDL, settled at the bottom of the tube. For statistical analysis, total lipoprotein intensity and fractional intensities of each region were determined by measuring the area under the curve (AUC) of the entire fluorescence trace and each region, respectively. In addition, each AUC value was normalized by the total AUC and presented as AUC%.

II.2.6 Electron microscopy

Transmission electron microscopy (TEM) has an advantage of greater resolution of the ultrastructure of biological and inorganic specimens. TEM consists of an electron emission source, electromagnetic lenses and an electron detector. Unlike light microscopes, electron

microscope uses a beam of electrons generated by electron gun to form an image. Magnification and focus are obtained by electromagnetic lenses because glass lenses for light microscopes would impede electrons. [82,83]

One blood sample was obtained from a healthy dog. The protocol and AUP (IACUC2014-0109CA) mentioned above were used for the blood draw. Written informed consent was obtained from the owner of the enrolled dog. Food had been withheld from the dog for at least 12 hours prior to the blood collection. In addition, a blood sample was obtained from a healthy cat. The study protocol was approved by the IACUC of Texas A&M University (IACUC2017-0190CA). Food was not withheld for the purpose of this blood collection. The cat was fed commercial dry food one hour prior to the blood collection. The blood samples were collected in serum tubes without any additives and left at room temperature for at least 40 minutes. The samples were then centrifuged for 15 minutes at 1,600 × g and 20°C. The serum samples were stored at 4°C until analysis. Ten µL of serum sample and 1280 µL of the NaBiEDTA density solution were mixed well. The sample solution was centrifuged at 867,747 × g for 6 hours at 4°C. After centrifugation, nominal LDL/HDL and HDL ranges (1.0191-1.0629 g/mL and 1.0632-1.178 g/mL, respectively) were manually removed from the top of the tube by a pipette based on the tube coordinate (mm) corresponding to nominal LDL/HDL and LDL (9.1-22.7 mm, and 22.7-29.0 mm, respectively). Each fraction was desalted using a 0.15 M sodium chloride solution and a centrifugal filter, Amicon® Ultra-0.5mL (MilliporeSigma, Billerica, MA). After desalting, the fractions were stored at 4°C until further analysis. Each fraction of canine serum was negatively stained by 2% of phosphotungstate and the grids were examined with a transmission electron microscope, Philips Morgagni 268 TEM (FEI, Hillsboro, OR). The

diameters of 550 particles were measured by an image processing program, ImageJ (public domain).

II.3 Results

II.3.1 Assay repeatability and reproducibility

Table 1 presents the %CVs for intra- and inter-assay variability for both AUC and AUC% for all 3 density ranges (TRL, LDL/HDL, and LDL). The highest %CV for intra-assay variability was 11.2%, while the highest %CV for inter-assay variability was 21.4%.

	Intra-assay		Inter-assay	
	AUC	%CV(AUC%)	%CV(AUC)	%CV(AUC%)
TRL	11.2	8.8	21.4	19.8
LDL/HDL	7.3	3.1	10.6	8.0
HDL	4.6	0.8	9.3	3.7

Table 1 %CVs for intra-and inter-assay variability for three density ranges: TRL, nominal LDL/HDL, and nominal HDL. AUC=area under the curve; CV= coefficient of variation; HDL= high density lipoproteins; LDL= low density lipoproteins; TRL=triglyceride rich lipoproteins

II.3.2 Effect of freeze-thaw cycles

For the density range of TRL, the %CVs for the samples thawed up to three times after storing at -80°C were relatively high (median [min-max]: 23.7 [2.3-39]%: Table 2). For the density range of LDL/HDL, the %CVs for the samples thawed up to three times after storing at -

80°C were less than 17.7 % (Table 3). For the density range of HDL, the %CVs for the samples thawed up to three times after storing at -80°C were less than 6.7% (Table 4).

	A		**B**		**C**		**D**	
TRL	AUC	AUC%	AUC	AUC%	AUC	AUC%	AUC	AUC
-80°, thawedx1	17.7	22.6	2.3	5.2	30.0	31.1	28.7	29.0
-80°C, thawedx2	23.9	20.6	23.9	22.0	11.0	9.5	24.3	19.4
-80°C, thawed x3	26.2	27.2	10.5	9.1	39.0	38.1	23.6	26.3

Table 2 The %CVs for the effect of freeze-thaw cycles for the density range of TRL under different storage conditions. A, B, C, and D represent four different serum samples from four healthy dogs.

	A		**B**		**C**		**D**	
LDL/HDL	AUC	AUC%	AUC	AUC%	AUC	AUC%	AUC	AUC%
-80°C, thawedx1	9.3	2.2	1.9	7.0	13.8	11.4	15.7	15.1
-80°C, thawedx2	3.4	1.5	6.4	2.3	2.0	2.9	2.8	3.0
-80°C, thawedx3	1.6	0.9	6.3	3.0	17.7	16.1	15.2	11.6

Table 3 The %CVs for the effect of freeze-thaw cycles for the density range of nominal LDL/HDL under different storage conditions. A, B, C, and D represent four different serum samples from four healthy dogs.

	A		B		C		D	
HDL	AUC	AUC%	AUC	AUC%	AUC	AUC%	AUC	AUC%
-80°C, thawedx1	6.7	1.1	4.8	3.0	4.3	1.5	0.4	0.7
-80°C, thawedx2	5.4	0.6	1.6	2.2	3.1	0.7	5.0	0.8
-80°C, thawedx3	3.5	1.2	2.4	1.2	2.6	1.6	6.5	0.1

Table 4 The %CV for the effect of freeze-thaw cycles for the density range of nominal HDL under different storage conditions. A, B, C, and D represent four different serum samples from four healthy dogs.

II.3.3 Stability of lipoproteins in canine serum samples

Table 5 and 6 present the %CVs for samples measured at day 0 and at various time points after storage at 4°C or -20°C for 3 days, 1 week, and 1 month. The %CVs for the density range of TRL were less than 35%, the %CVs for the density range of LDL/HDL were less than 19.5%, and the %CVs for the density range of HDL were less than 9.1%. Table 7 presents the %CV for samples measured at day 0 and various time points after storing at -80°C for 3 days, 1 week, and up to 12 months. Under these conditions, the %CVs for the density ranges of TRL, LDL/HDL, and HDL were <24.6%, <13.9%, and <7.7%, respectively.

	E		F		G	
	%CV(AUC)	%CV(AUC%)	%CV(AUC)	%CV(AUC%)	%CV(AUC)	%CV(AUC%)
TRL	14.3	11.7	23.8	20.8	20.1	19.3
LDL/HDL	8.9	7.0	14.2	10.6	19.5	16.4
HDL	3.4	3.8	3.3	5.2	5.4	6.0

Table 5 %CVs for stability of lipoprotein profiles (AUC and AUC%) for samples stored up to 1 month at 4°C. E, F, and G represent three different serum samples from three different dogs. AUC=area under the curve; CV= coefficient of variation; HDL= high density lipoproteins; LDL= low density lipoproteins; TRL=triglyceride rich lipoproteins

	E		F		G	
	%CV(AUC)	%CV(AUC%)	%CV(AUC)	%CV(AUC%)	%CV(AUC)	%CV(AUC%)
TRL	14.1	5.5	6.8	11.5	35.0	31.2
LDL/HDL	15.2	7.3	4.8	2.5	8.2	7.7
HDL	9.1	3.7	7.0	1.3	8.7	2.8

Table 6 %CVs for stability of lipoprotein profiles (AUC and AUC%) for samples stored up to 1 month at -20°C. E, F, and G represent three different serum samples from three different dogs. AUC=area under the curve; CV= coefficient of variation; HDL= high density lipoproteins; LDL= low density lipoproteins; TRL=triglyceride rich lipoproteins

	E		F		G	
	%CV(AUC)	%CV(AUC%)	%CV(AUC)	%CV(AUC%)	%CV(AUC	%CV(AUC%)
TRL	18.5	18.8	16.1	16.0	21.5	24.6
LDL/HDL	13.9	7.3	11.2	2.5	11.6	7.7
HDL	7.7	3.5	5.0	3.3	5.9	1.7

Table 7 %CVs for stability of lipoprotein profiles (AUC and AUC%) for samples stored up to 12 months at -80°C. E, F, and G represent three different serum samples from three different dogs. AUC=area under the curve; CV= coefficient of variation; HDL= high density lipoproteins; LDL= low density lipoproteins; TRL=triglyceride rich lipoproteins

II.3.4 Electron Microscopy

II.3.4.1 Canine lipoprotein particles

Electron microscopy examination showed the spherical particles in both density ranges. The particles within the density range of LDL/HDL ranged in size from 9.5-29.0 nm (median: 16.1 nm; Figure 1). The particles within the density range of HDL ranged in size from 9.7-17.2 nm (median: 13.2 nm; Figure 2). Figure 3-4 presents the distribution of particle diameters. Figure 3 shows that approximately 52% of particles within the density range of LDL/HDL were between 11.0 and 17.0 nm in diameter, while 47% of particles within the same range were between 17.0 and 30.0 nm in diameter. Almost all particles within the density range of HDL were between 10 and 17.5nm in diameter (Figure 4).

II.3.4.2 Feline lipoprotein particles

Electron microscopy examination showed spherical particles in both density ranges. The particles within the density range of nominal LDL (1.0191-1.0629 g/mL) ranged in size from 7.6 to 34.6 nm (median: 12.6 nm; Figure 5 and 6). The particles within the density range of nominal

HDL (1.0632-1.178 g/mL) ranged in size from 8.3 to 16.2 nm (median: 9.9 nm; Figure 7 and 8). Approximately 83% of lipoproteins within the LDL range were 9-16 nm in diameter, while more than 90% of lipoproteins within the HDL range were 9-11 nm in diameter.

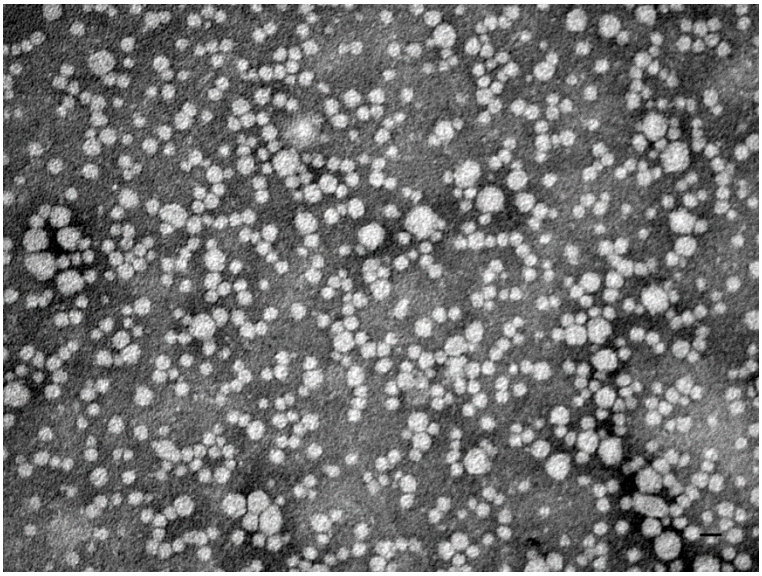

Figure 1 Negatively-stained (2% phosphotungstate) canine lipoproteins of the density range of nominal LDL (1.0191-1.0629 g/mL). Bar=20nm.

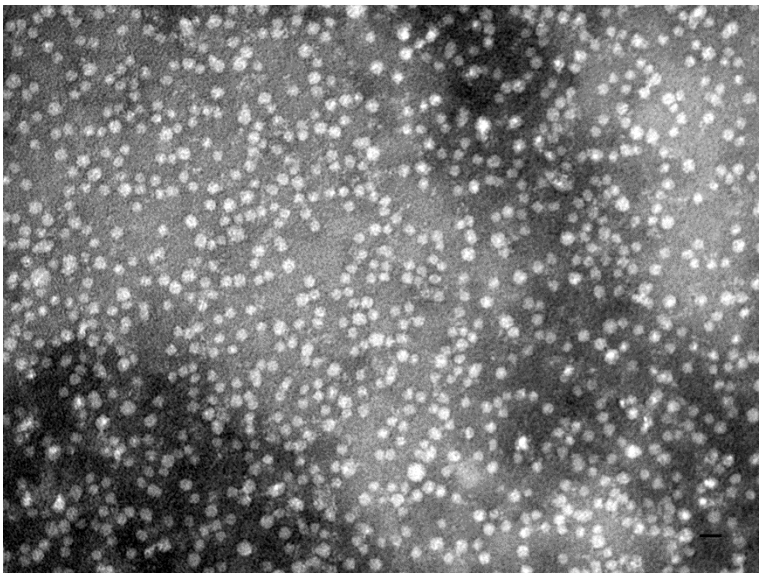

Figure 2 Negatively-stained (2% of phosphotungstate) canine lipoproteins of the density range of nominal HDL (1.0632-1.178 g/mL). Bar =20 nm.

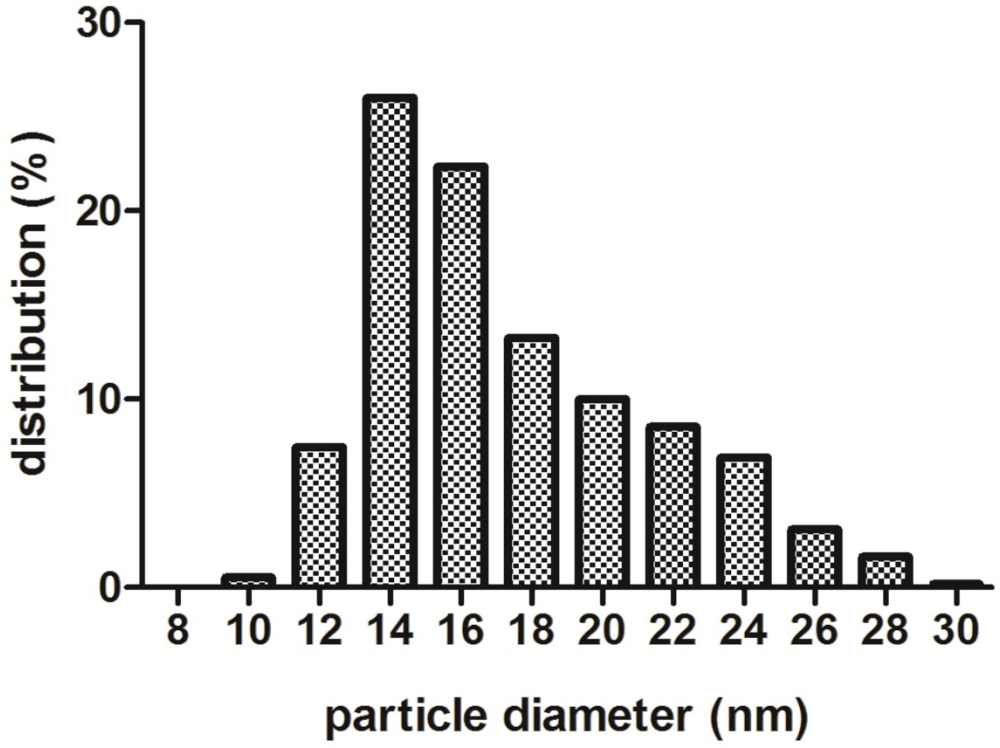

Figure 3 The distribution of diameters (nm) of canine lipoproteins within the density range of nominal LDL/HDL (1.0191-1.0629 g/mL).

The diameters of 550 particles from each density range were measured.

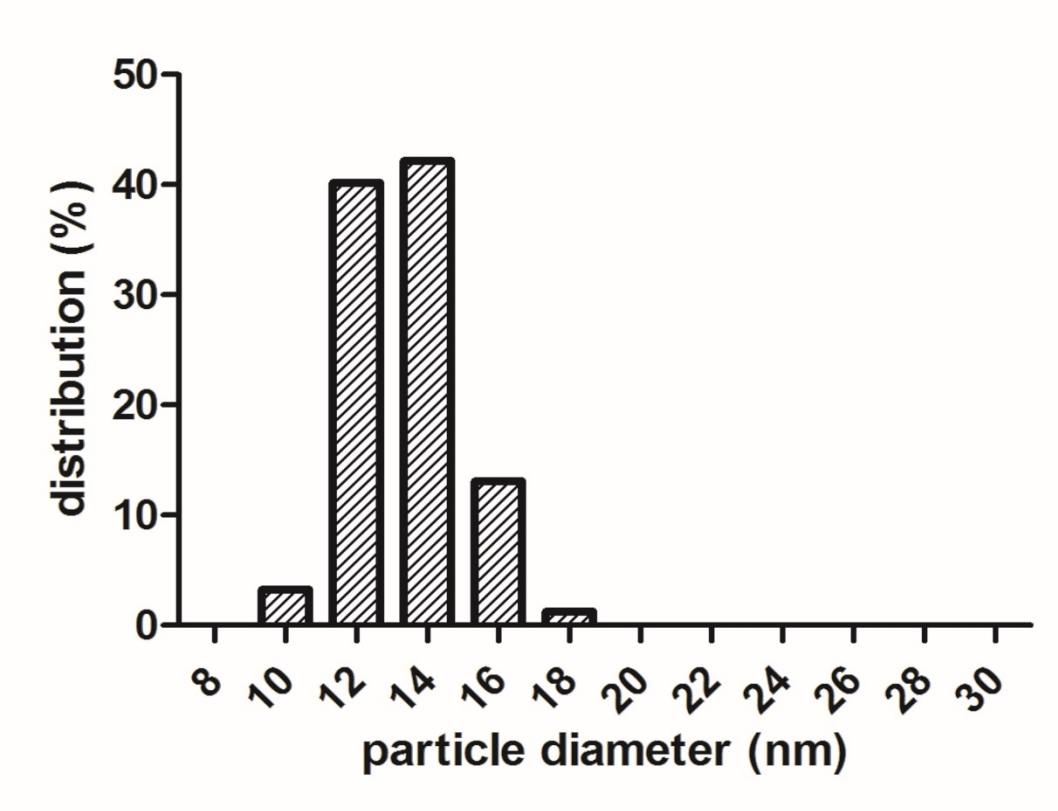

Figure 4 The distribution of diameters (nm) of canine lipoproteins within the density range of nominal LDL/HDL (1.0191-1.0629 g/mL).

The diameters of 550 particles from each density range were measured.

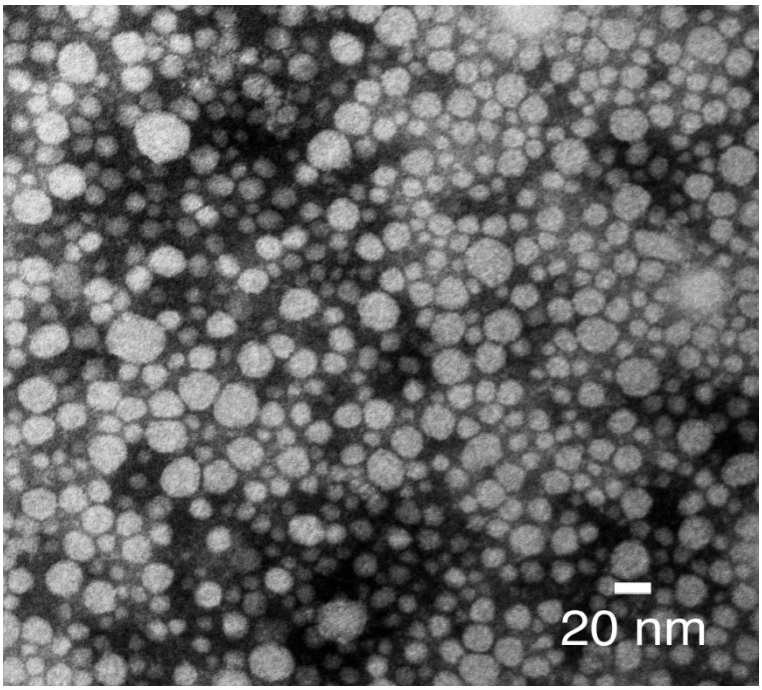

Figure 5 Negatively-stained (2% phosphotungstate) feline lipoproteins of the density range of nominal LDL (1.0191-1.0629 g/mL).

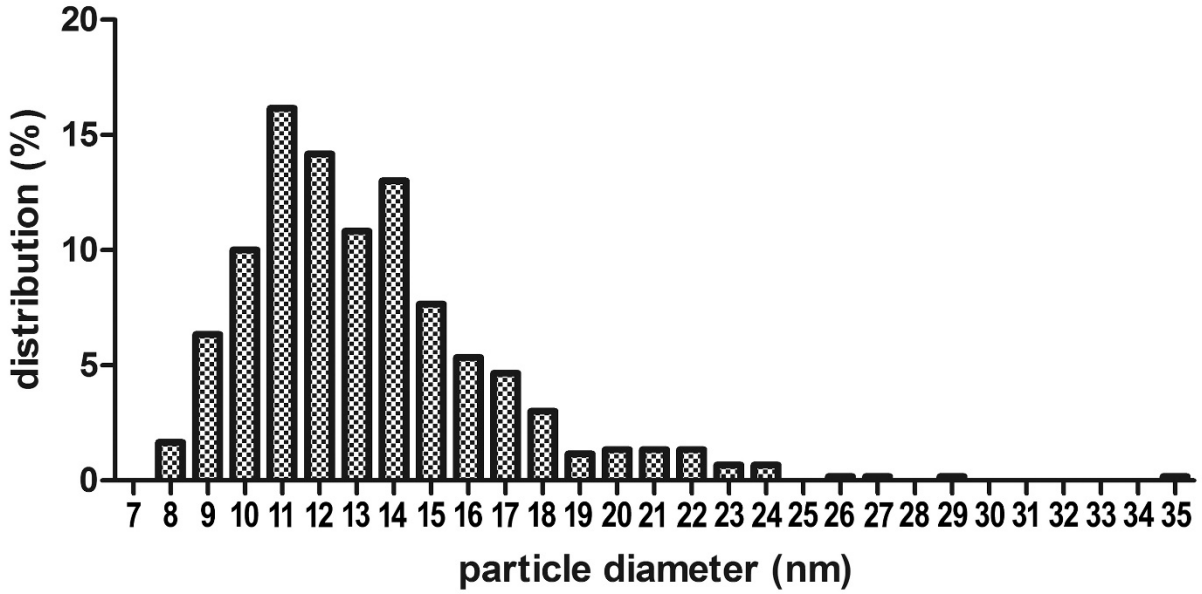

Figure 6 The distribution of diameters (nm) of feline lipoproteins within the density range of nominal LDL (1.0191-1.0629 g/mL).

The diameters of 600 particles from each density range were measured.

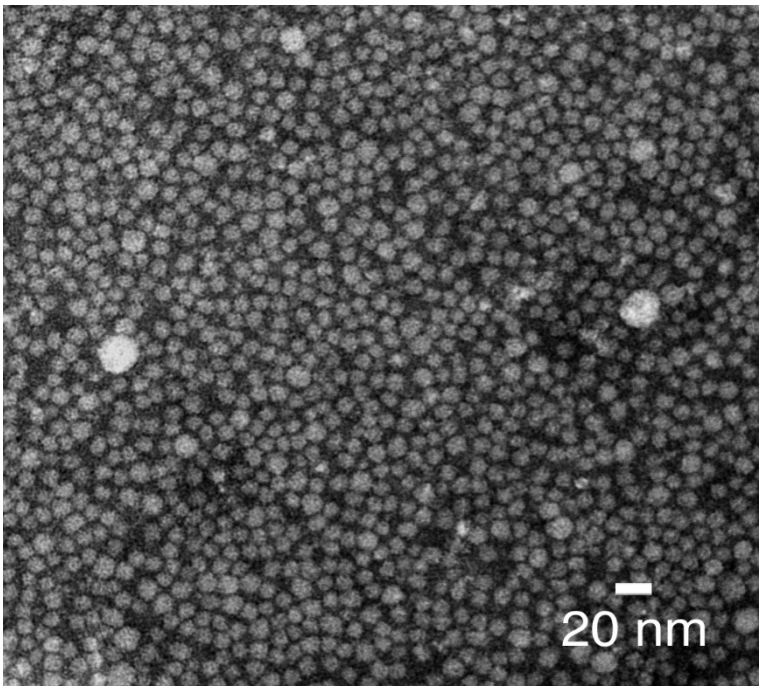

Figure 7 Negatively-stained (2% phosphotungstate) feline lipoproteins of the density range of nominal HDL (1.0632-1.178 g/mL).

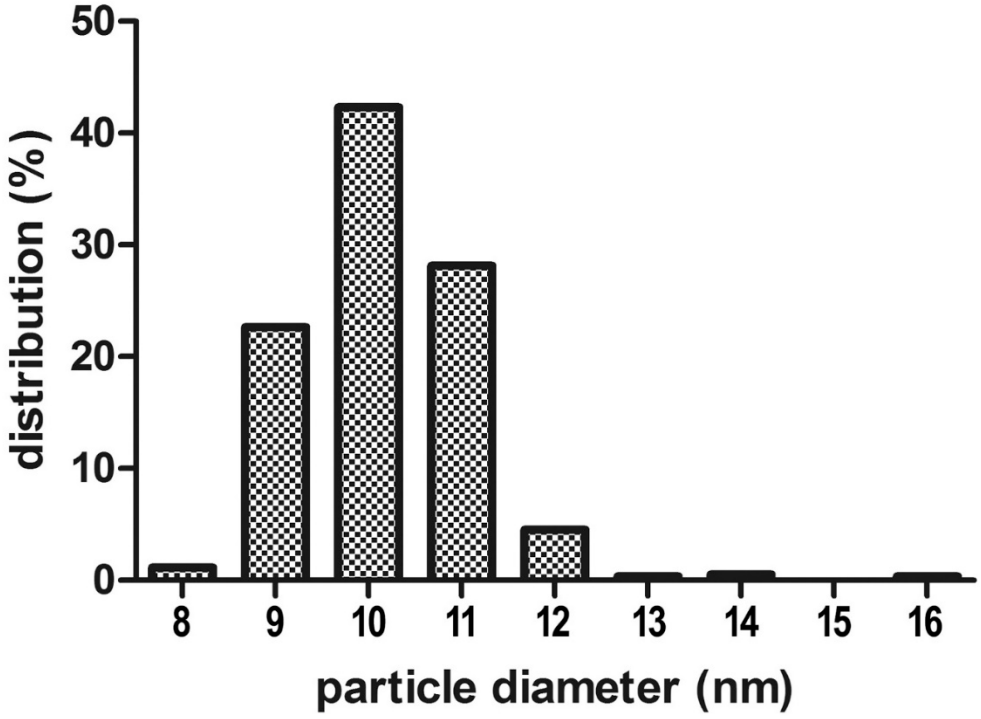

Figure 8 The distribution of diameters (nm) of feline lipoproteins within the density range of nominal HDL (1.0632-1.178 g/mL).

The diameters of 600 particles from each density range were measured.

II.4 Discussion

The CLPDP method was initially developed to analyze lipoprotein profiles in human plasma samples based on human lipoprotein density distribution.[74] For partial validation of the CLPDP technique for use with canine serum samples, we performed intra- and inter- assay validation using one pooled canine sample. The intra- and inter- assay variability showed that the CLPDP was repeatable and reproducible for the density ranges of nominal LDL/HDL and HDL, whereas the method was repeatable but not reproducible for the density range of TRL. A larger sample size with range of lipoprotein concentrations (high and low) is needed to fully analytically validate the CLPDP. In addition, we evaluated the effect of the freeze-thaw cycles on lipoprotein profiling as well as the stability of lipoprotein profiles under different storage conditions for different storage time periods. The effect of up to three freeze-thaw cycles after storing at -80°C was acceptable for the density ranges of nominal LDL/HDL and HDL. However, storing at -80°C may not be suitable for evaluating the density range of TRL and fresh samples may be required. We also found that lipoproteins in canine serum which are in the range of nominal LDL/HDL and HDL are stable for up to 1 month at 4°C and -20°C as well as for up to 12 months when stored at -80°C. These findings suggest that sample handling, storage condition, and freeze-thaw cycles in this study did not affect the assessment of lipoprotein profiles and were unlikely to affect the results collected from clinical samples.

Canine HDL and LDL overlap in hydrated density, making it impossible to separate them completely based on their density.[78,79] Therefore, the lipoprotein classification based on their density distribution utilized in human samples does not precisely represent the canine lipoprotein distribution. In order to investigate canine lipoprotein distribution by the CLPDP, we examined the shapes and sizes of canine lipoproteins in the density ranges of nominal LDL/HDL and HDL

with an electron microscope. Negative-staining electron microscopy showed that spherical particles in both density ranges. Approximately 52% of particles within the density range of nominal LDL/HDL (1.0191-1.0629 g/mL) were between 11.0 and 17.0 nm in diameter, while 47% of particles within the same range were between 17.0 and 30.0 nm in diameter. Almost all particles within the density range of nominal HDL (1.0632-1.1781 g/mL) were between 10 and 17.5 nm in diameter. These results are similar to the previous reports on human and canine lipoprotein diameters.[75,78] This finding suggests that there is overlap of particle diameters between the two density ranges distributed by the CLPDP, but the density range of LDL showed bigger particles, which is one of characteristics of LDL particles.

Electron microscopic examination also showed that feline lipoproteins were spherical and the diameters of the majority of lipoproteins that fell into the LDL density range were larger than those that fell into the HDL density range as has also been reported in humans and dogs. [75,78] In addition, feline lipoproteins in this study were smaller in diameter than what has previously been reported for lipoproteins in humans and dogs.[75,78] These findings suggest that the CLPDP separates feline lipoproteins well and can be used to evaluate lipoprotein density distributions in feline serum samples. We examined serum lipoproteins with TEM only from one healthy cat, thus this result might not represent lipoprotein sizes in other healthy cats. A larger sample size is needed to further investigate average diameters of feline lipoproteins.

CHAPTER III

ANALYTICAL ASSESSMENT OF SERUM LIPOPROTEIN PROFILES IN HEALTHY DOGS AND DOGS WITH EXOCRINE PANCREATIC INSUFFICIENCY

III.1 Introduction

Lipoproteins transport lipids in the bloodstream and are often classified based on their hydrated density. In humans, these density classes include the intestinally derived chylomicrons, hepatically assembled very low density lipoproteins (VLDL), and their lipolytic by-products: intermediate density lipoproteins (IDL) and low density lipoproteins (LDL), as well as peripherally formed high density lipoproteins (HDL).[84] In the human literature, altered proportions of lipoprotein classes have been described in certain disease states, such as coronary artery disease .[8] In veterinary medicine, hyperlipidemia is a general term associated with various diseases and can cause clinically relevant complications, such as cutaneous xanthomas,[18] liver disease,[19] cholelithiasis,[20] pancreatitis,[21] glomerular disease,[22] lipemia retinalis, or peripheral neuropathy.[23] However, limited published reports are available in veterinary medicine regarding characteristics of lipoproteins in diseased animals.

Exocrine pancreatic insufficiency (EPI) is a syndrome caused by the insufficient synthesis and secretion of pancreatic enzymes.[85,86] The pancreatic enzymes contribute to digestion of dietary lipid (e.g., lipase and phospholipase), proteins (e.g., trypsin and chymotrypsin), and carbohydrates (amylase), and other macronutrients (e.g., elastase, DNAse and RNAse).[87] The most common causes of EPI in dogs are acinar atrophy or chronic pancreatitis, resulting in an absolute lack of pancreatic acinar cells.[85] It has been estimated that more than 90% of the functional capacity of the exocrine pancreas must be lost before clinical

signs develop.[86] Clinical signs include weight loss, increased appetite, loose stool, and a poor hair coat. EPI is treated with pancreatic enzyme replacement therapy.[85,86] Thus, we hypothesized that 1) dogs with EPI have dyslipidemia due to the lack of lipase, phospholipase, and cholesterol esterase, which are necessary to digest and absorb the ingested fats (triglycerides and cholesterol), 2) enzyme replacement therapy in dogs with EPI would improve dyslipidemia.

The aims of this study was to compare the lipoprotein profiles using the CLPDP, and serum cholesterol and triglyceride concentrations between 29 healthy dogs, dogs with EPI that had been treated with enzyme supplementation (EPI-T: n=22), and those not yet treated (EPI-NT: n=6) at the time of the sample collection.

III.2 Materials and methods

III.2.1 Healthy dogs

Surplus serum samples from 29 privately owned dogs whose owners had volunteered them for other IACUC approved studies were identified. Written informed consent had been obtained from all owners of enrolled dogs. Food had been withheld from these dogs for at least 12 hours prior to the blood collection. In order to determine the dog's health status, physical examination, a serum chemistry profile and a complete blood count were performed. Originally, the blood samples were collected in serum tubes without any additives and left for at least 40 minutes at room temperature. The samples were then centrifuged for 15 minutes at 1,600 × g and 20°C. The serum fraction was separated and stored at -80°C until lipoprotein profiling. The CLPDP (lipoprotein profiling) was performed within 3 months.

III.2.2 Dogs with EPI

Serum samples were obtained from 28 dogs with EPI that were enrolled in a related IACUC approved project. Written informed consent had been obtained from all owners of enrolled dogs. EPI was confirmed with serum trypsin-like immunoreactivity (TLI) concentration ≤ 2.5 µg/L. All enrolled dogs with EPI had serum cobalamin concentrations ≥ 400 ng/L for the initial study's criterion not for this study. Other inclusion criteria included age of more than one year, presence of one or more clinical signs of EPI (e.g., polyphagia, weight loss, steatorrhea, and/or loose, voluminous, and/or malodorous stools), and no evidence of any additional disease processes based on history, physical examination, and laboratory analysis. Food had been withheld from these dogs for at least 12 hours prior to the blood collection. Serum samples were shipped to the Gastrointestinal Laboratory at Texas A&M University overnight with icepacks. Laboratory analysis, including TLI, was performed on the next working day, this meant within 4 days of collection. Surplus serum samples were aliquoted and stored at -80°C until further analysis. Lipoprotein profiles were analyzed using the CLPDP within 3 months. The dogs with EPI were categorized into two groups: those already being treated with enzyme supplementation (EPI-T: n=22) and those not yet treated (EPI-NT: n=6) at the time of blood sample collection.

III.2.3 Continuous lipoprotein density profiling (CLPDP)

The CLPDP was carried out as described previously with some modifications.[56,81] Briefly, 1280 µL of 0.18 M NaBiEDTA density solution (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was mixed with 10 µL of serum and 10 µL of NBD C6-ceramide (Cayman Chemical Company, Ann Arbor, MI). Then 1150 µL of the mixture was transferred into a polycarbonate centrifuge tube (Beckman Coulter, Brea, CA). The sample tubes were centrifuged

at 867,747 × g and 4°C for 6 hours in Optima MAX-LP ultracentrifuge (Beckman Coulter, Brea, CA) with a fixed angle MLA-130 rotor (Beckman Coulter). After centrifugation, the tubes were immediately imaged by a fluorescence imaging system consisting of a digital camera (Quantifire XI, Optronics, Muskogee, OK) and a metal halide continuous light source (Dolan-Jenner Industries, Boxborough, MA). Following ultracentrifugation, the image of each tube was converted to a density profile using a software program, OriginPro7.5 (OriginLab, Northampton, MA). The tube coordinate (mm) on the X-axis of the lipoprotein profile corresponded to an actual centrifuge tube coordinate (mm). Zero mm indicated the top of the tube, and 33 mm indicated the bottom of the tube. The average intensity of fluorescence was plotted on the Y-axis to produce a lipoprotein profile. The less dense particles, such as triglyceride-rich lipoproteins (TRL), migrated near the top of the tube, whereas more dense particles, such as HDL, settled at the bottom of the tube. For statistical analysis, total lipoprotein intensity and fractional intensities of each region were determined by measuring the area under the curve (AUC) of the entire fluorescence trace and each region, respectively. In addition, each AUC value was normalized by the total AUC and presented as AUC%.

II.2.4 Nomenclature

For statistical analysis, the numbering nomenclature from subfractions #2 to #11 was created based on every 2 mm (tube coordinate) from the top of the tube except subfraction #1. Subfraction #1 was determined based on the first peak (1.0102-1.0190 g/mL), which corresponds to TRL, such as chylomicrons and VLDL. Subfractions between #2 and #8 correspond to nominal LDL/HDL (1.0191-1.0629 g/mL), and subfractions between #9 and #11 correspond to

nominal HDL (1.0632-1.1781 g/mL). Total lipoprotein intensity was also determined by measuring the AUC of entire fluorescence trace and presented as a total AUC.

III.2.5 Serum triglyceride and cholesterol concentrations

Serum cholesterol and triglyceride (TG) concentrations were measured using a clinical chemistry analyzer (SIRRUS, STANBIO, Boerne, TX).

III.2.6 Lipoprotein metabolism index

An index, termed lipoprotein metabolism index was created to aid interpretation of the results by calculating the ratio of #10 (AUC%) to #3 (AUC%). Subfraction #3 corresponds to the density range of 1.0230-1.0272 g/mL, which corresponds most likely to apoB containing lipoprotein particles, such as VLDL and LDL.[56] Subfraction #10 corresponds to the density range of 1.0858-1.1190 g/mL, which corresponds most likely to apolipoproteinA-I (apoAI) containing lipoprotein particles (HDL).[56,75] Thus, the lipoprotein metabolism index indicates the ratio between VLDL/LDL and HDL.

III.2.7 Statistics

The distribution of the data was assessed using the Shapiro-Wilk test and visual inspection. A nonparametric test (Wilcoxon rank sum test) and nonparametric comparisons for each pair using Wilcoxon method were used. Significance was set at $p < 0.05$. The Benjamini-Hochberg False Discovery Rate (FDR) was used to calculate adjusted *p*-value (*q*-value) for multipule comparisons where appropriate. Significance was set at $q < 0.05$. Metabolomic data analysis software, MetaboAnalyst 3.0[88] was used to generate a principal component analysis (PCA) plot in order to show the clustering/overlap among lipoprotein profiles based on %AUCs of 11 subfractions in healthy dogs, dogs in the EPI-T group, and dogs in the EPI-NT group.

III.3 Results

III.3.1 Study population

Table 8 shows breed, sex, weight (kg), and age of the enrolled dogs. None of the parameters differed significantly between healthy control dogs, dogs in the EPI-T group, or dogs in the EPI-NT group.

signalment	Healthy	EPI	
		EPI-NT	EPI-T
Pure breed, n (number of breeds)	19 (12)	6 (3)	17 (10)
Mixed breed, n	9	0	5
Males, n (neutered)	12 (8)	3(1)	8 (5)
Females, n (spayed)	17 (15)	3 (3)	14 (12)
Weight, median (range) kg	27.2 (2.7-40.1)	24.7 (9.7-29.4)	24.7 (2.7-37.5)
Age, median (range) year	4 (1-12)	2.7 (1-4)	2.7 (1-14)

Table 8 Signalment of healthy control dogs, dogs in the EPI-T group, and dogs in the EPI-NT group.

EPI=exocrine pancreatic insufficiency; EPI-NT=EPI without enzyme supplementation; EPI-T=EPI already treated with enzyme supplementation; n=number of dogs

III.3.2 Serum TG and cholesterol concentrations

Serum TG concentrations in healthy control dogs were significantly higher than those in dogs in the EPI-NT group (median [min-max]: 53 mg/dL [34-585 mg/dL] vs. 41 mg/dL [24-51 mg/dL], p=0.0054). Serum cholesterol concentrations in dogs in the EPI-NT group (median [min-max]: 123.5 mg/dL [71-160 mg/dL]) were significantly lower than those in the healthy control dogs (median [min-max]: 240 mg/dL [129-368 mg/dL], p=0.0004) or dogs in the EPI-T group (median [min-max]: 206.5 mg/dL [76-326 mg/dL], p=0.0127).

III.3.3 Lipoprotein profiles

Figures 9, 10, and 11 are overlay graphs for healthy control dogs, dogs in the EPI-T and dogs in the EPI-NT groups, respectively. As mentioned above, subfraction #1 corresponds to TRL, subfractions between #2 and #8 correspond to nominal LDL/HDL (1.0191-1.0629 g/mL), subfractions between #9 and #11 to nominal HDL (1.0632-1.1781 g/mL) .

Total AUC and the AUCs for subfractions from #1 to #10 differed significantly between all three groups (healthy control dogs, dogs in the EPI-T, and dogs in the EPI-NT group; q< 0.05), while subfraction #11 did not differ significantly between those three groups (q=0.125). Of the subfractions from #1 to #10 except #4 and #8, AUCs in healthy dogs were the highest followed by dogs in the EPI-T and then dogs in the EPI-NT groups (healthy dogs > EPI-T > EPI-NT). The AUC% for 9 subfractions (2-8, 10, and 11) differed significantly between the three groups (q < 0.05), while the AUC% for 2 subfractions, 1 and 9, did not differ significantly between all three groups (q = 0.148, q = 0.081, respectively). Moreover, the AUC% for subfractions from #3 to #8 and #10 significantly differed between healthy dogs and dogs in the EPI-NT (q < 0.05), and between dogs in the EPI-T group and dogs in the EPI-NT group (q <

0.05), but not between healthy dogs and dogs in the EPI-T group ($q < 0.05$). The lipoprotein metabolism index (ratio of #10/#3; Figure 12) differed significantly between the EPI-T and EPI-NT groups ($p = 0.0173$), and between healthy control dogs and the EPI-NT group ($p = 0.0041$). The PCA plot (Figure 13) shows the distribution of each sample based on their similarity of lipoprotein profile composition (%AUC for a total of 11 subfractions). Visual separation could be seen with a shift from the right upper to bottom left side of the PCA plot that correspond to the differences between healthy control dogs, dogs in the EPI-T group, and dogs in the EPI-NT group.

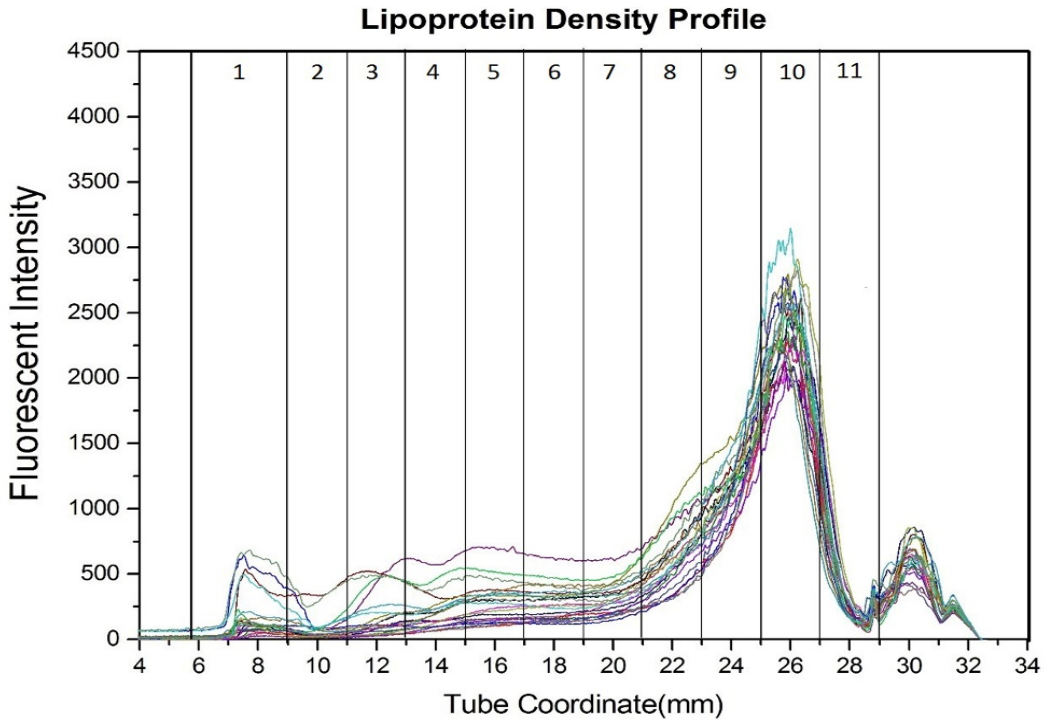

Figure 9 Overlay of lipoprotein profiles from 29 healthy control dogs.

The X-axis shows the tube coordinate (mm) and the Y-axis displays the fluorescent intensity. Most dogs had similar patterns characterized by a high peak at subfraction #10.

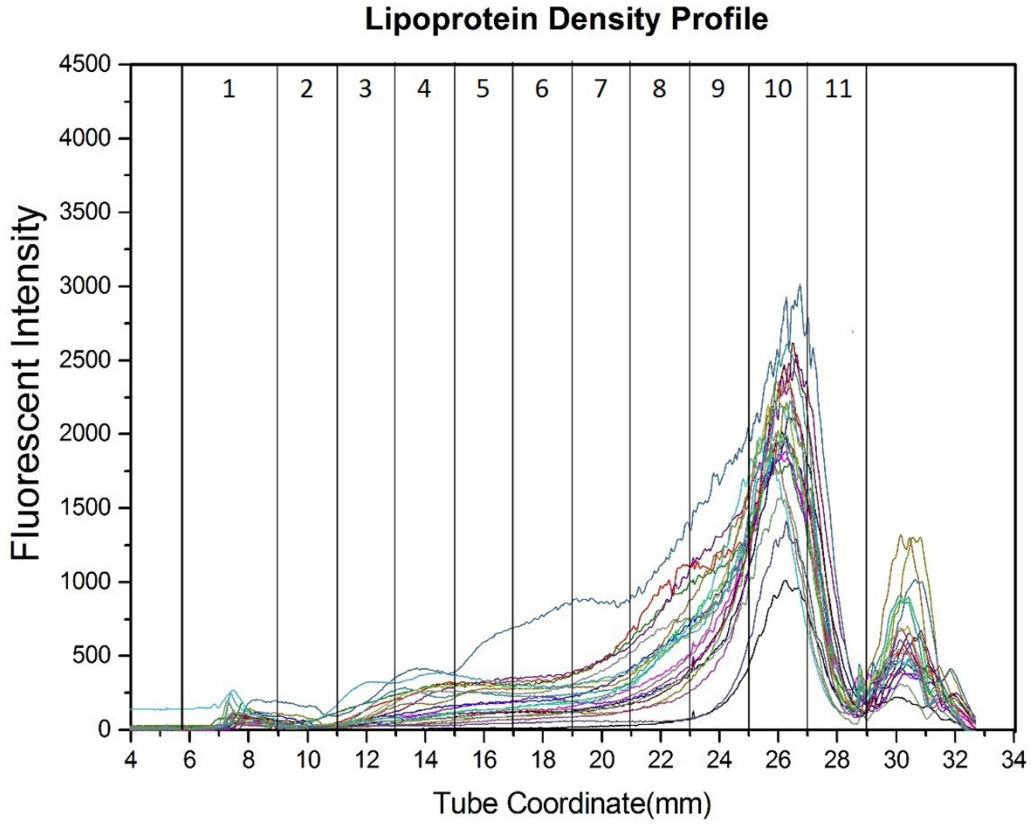

Figure 10 Overlay of lipoprotein profiles form dogs with EPI treated with enzyme supplementation (EPI-T). Some dogs had smaller peaks at subfraction #10 as well as between subfractions #4 and #9.

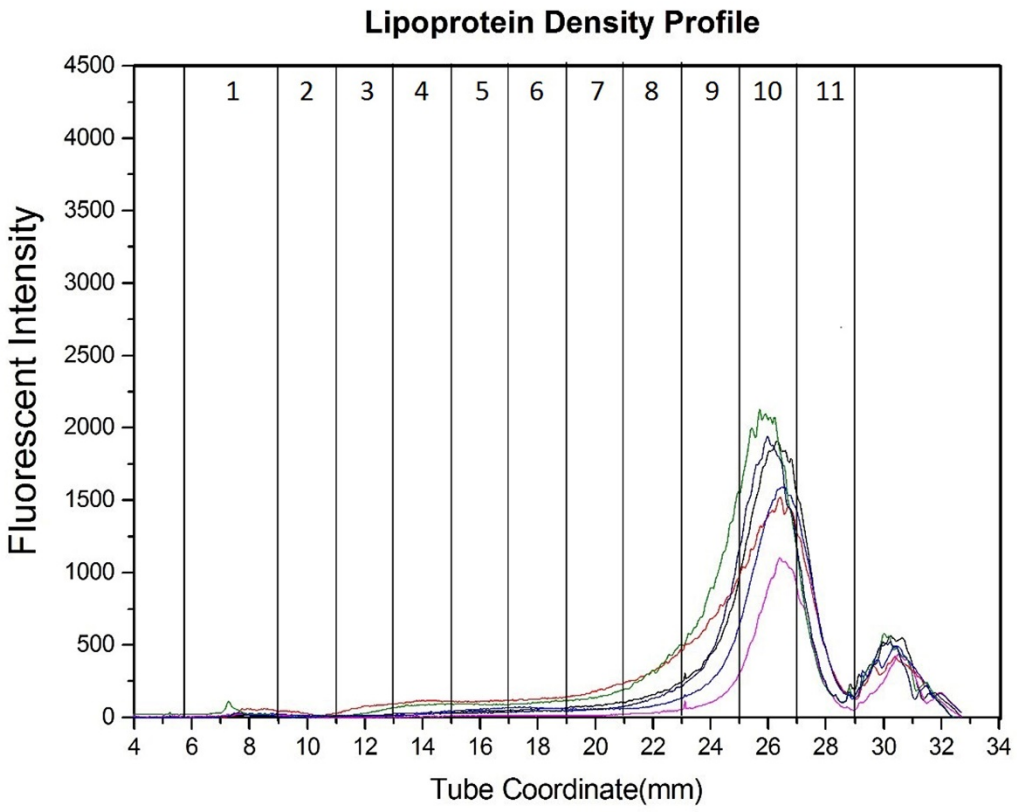

Figure 11 Overlay of lipoprotein profiles in dogs with EPI not yet treated (EPI-NT). The dogs in this group had smaller peaks over all.

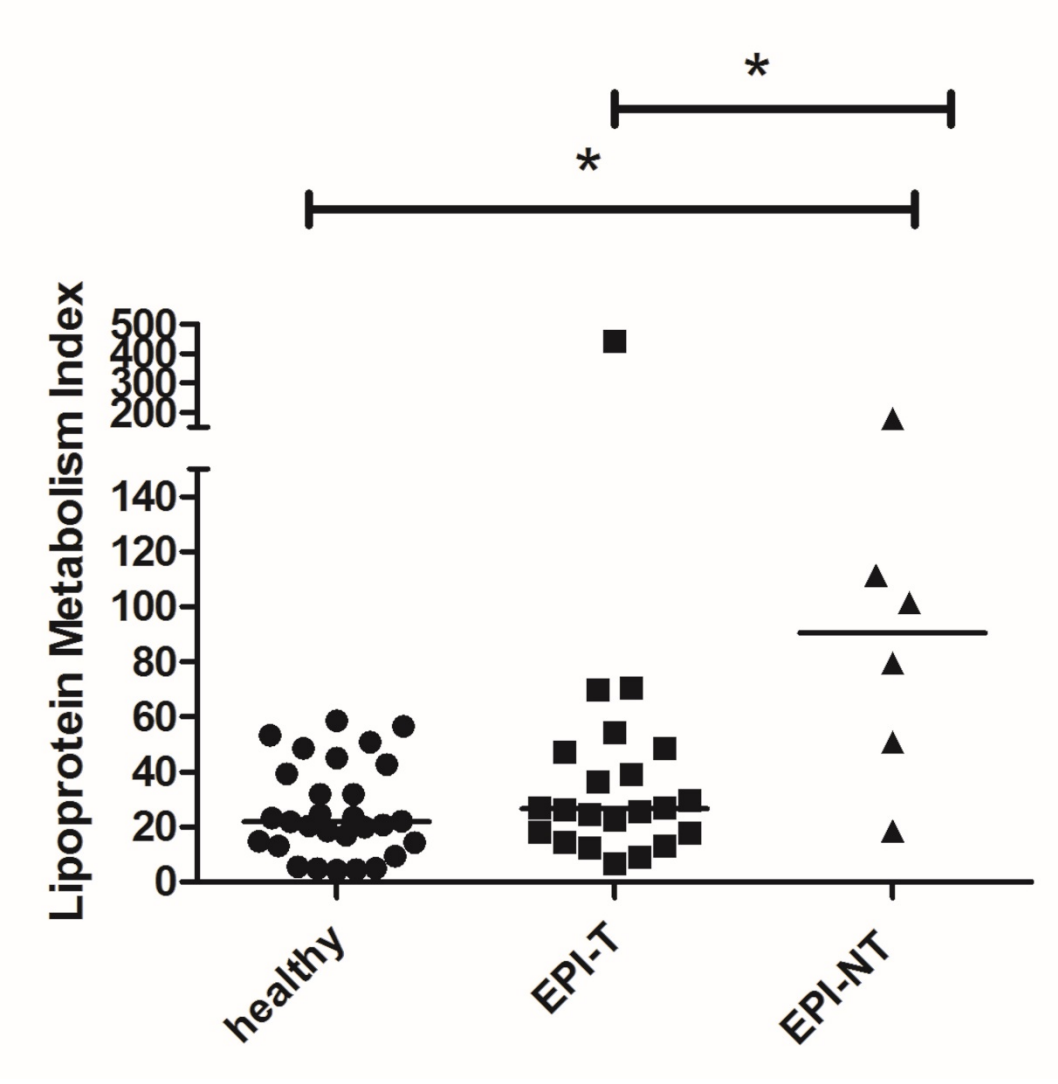

Figure 12 Lipoprotein metabolism index (ratio of fraction #10 to #3) between healthy control dogs, dogs with EPI treated with enzyme supplementation (EPI-T), and dogs with EPI not yet treated (EPI-NT).

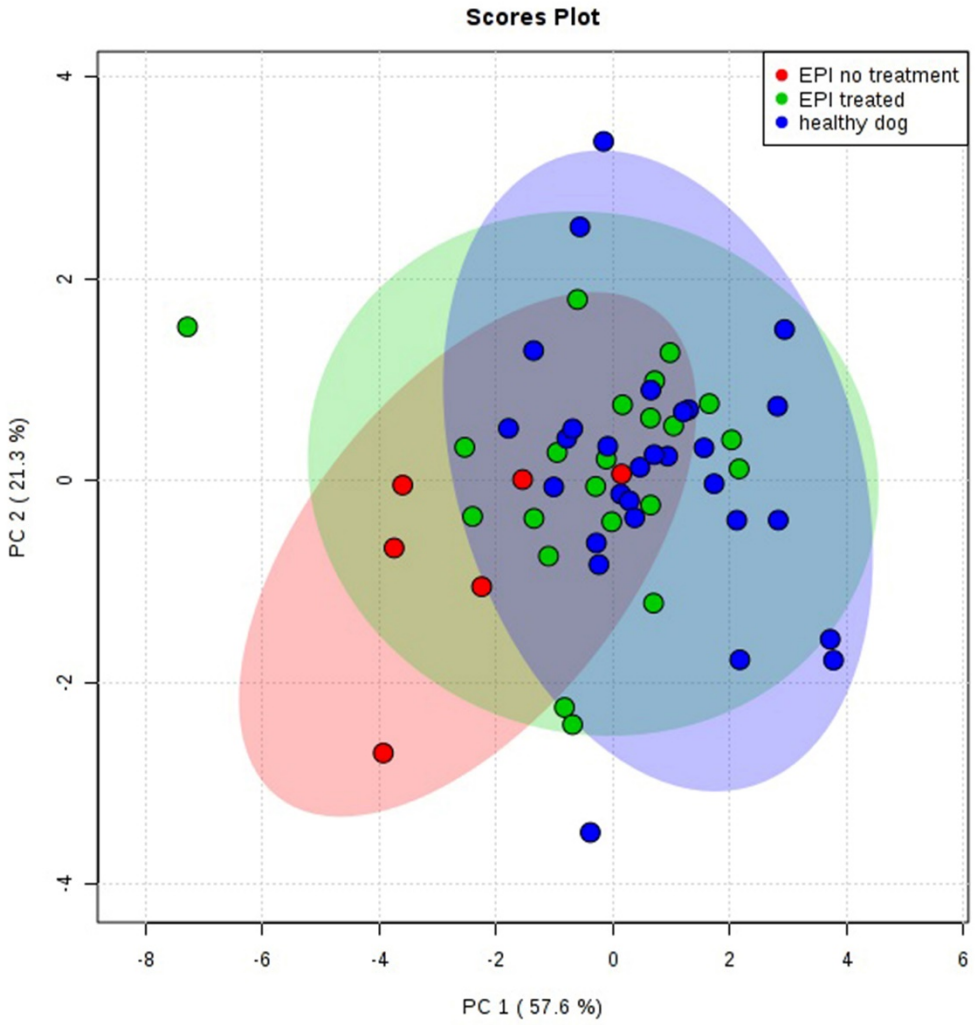

Figure 13 Principal component analysis (PCA) plot showing the relationship of lipoprotein profiles for healthy dogs (Blue) compered to dogs with EPI treated with enzyme supplementation (EPI-T: green) and those not yet treated (EPI-NT: red)

III. 4 Discussion

Serum TG concentrations in healthy control dogs were significantly higher than those in dogs in the EPI-NT group. Serum cholesterol concentrations in dogs with EPI that were not yet treated (EPI-NT) were significantly lower than those in healthy control dogs or dogs with EPI that were already treated (EPI-T). The lipoprotein profiles have shown that dogs with EPI in both the EPI-NT and EPI-T had significantly lower AUC andAUC% in most lipoprotein subfractions compared to healthy control dogs. When we looked at the lipoprotein metabolism index (ratio of #10/#3), dogs in the EPI-NT group had a significantly higher ratio than that of dogs in the EPI-T group or healthy control dogs. However, the lipoprotein metabolism index did not differ significantly between healthy control dogs and dogs in the EPI-T group. These results suggest that dogs with EPI have dyslipidemia as well as disproportionate levels of HDL (fraction #10) and VLDL (fraction #3) composed of significantly lower VLDL and HDL levels compared to healthy control dogs if they are not treated by enzyme supplementation. These findings can be also visually observed by comparing Figures 5, 6, and 7. The PCA plot suggests that dogs in the EPI-T group had similar lipoprotein profiles to those of healthy control dogs, whereas dogs in the EPI-NT group had altered lipoprotein profiles compared to healthy control dogs. However, even though these results suggest that enzyme supplementation therapy in dogs with EPI improves dietary lipid assimilation, both the lipoprotein profiles and PCA plot indicate that a few dogs under treatment had persistently altered lipoprotein profiles.

There are important lipoprotein metabolism pathways that might be involved in the pathogenesis of dyslipidemia in dogs with EPI. In normal physiological condition, dietary TGs are incorporated into chylomicrons in the enterocytes and released into the lymph. The main function of chylomicrons is to transport dietary TG into peripheral tissues as well as dietary

cholesterol to the liver. The chylomicron remnants eventually bind to the LDL receptor (LDL-R) and LDL-receptor-related protein (LRP), and are internalized into the hepatocytes. In the present study, dogs in the EPI-NT had significantly lower serum TG concentrations and TRL than those in healthy dogs. This result suggests that dogs with EPI have decreased serum TG because of insufficient absorption of dietary TG if they are not treated with enzyme supplementation. In contrast to the absorption process of dietary TG, endogenously synthesized TG and cholesterol are secreted within VLDL particles from the liver. VLDL remnants produced by TG hydrolysis in peripheral tissues are taken up by the liver and further metabolized by hepatic lipase to become LDL.[18] Decreased lipoproteins in the density range of VLDL and LDL in dogs with EPI might be explained by insufficient de novo fatty acid biosynthesis in the liver because of decreased absorption of carbon sources, such as glucose or amino acids from the intestines as well as decreased cholesterol ester uptake from chylomicron into the liver. Nascent HDL particles are secreted by the liver and small intestine, and acquire phospholipids and free cholesterol from peripheral tissues and TRL via phospholipid transfer protein (PLTP) with cholesteryl esters that formed via the action of lecithin-cholesterol acyltransferase (LCAT). Nascent HDL particles become larger and less dense HDL particles (HDL3 and HDL2). As mentioned above, because dogs with EPI had decreased TRL, their HDLs were most likely decreased because of insufficient supplies of phospholipids and free cholesterol from TRL.

The limitations of this study included a small sample size of the EPI-NT group. Enrolling additional dogs into the EPI groups would be required to make a more definitive statement regarding the efficacy of enzyme replacement therapy for the improvement of dyslipidemia.

The CLPDP might be a useful tool for evaluating dyslipidemia in dogs. Long-term observational studies are required to assess the relationship between clinical signs and lipoprotein profiles after enzyme replacement therapy in dogs with EPI.

CHAPTER IV

IPOPROTEIN PROFILING IN DOG WITH CHRONIC ENTEROPATHY OR HEPATIC DISEASE

IV. 1 Introduction

In the human literature, altered proportions of lipoprotein classes have been described in certain disease states, such as cardiovascular disorders,[7] metabolic derangements,[89] and inflammatory diseases, such as IBD[90,91] and systemic lupus erythematosus (SLE).[92,93] It has been shown that infection and chronic inflammation impair lipoprotein metabolism and cause a variety of changes in plasma concentration of lipids and lipoproteins in human patients.[92] The mechanisms of these alterations are not fully understood. However, several inflammatory mediators have been suggested to play a role in the alteration of lipid and lipoprotein metabolism. Inflammatory mediators include, but are not limited to, tumor necrosis factor alpha (TNF-α), interleukins (IL-1, 2, and 6), and interferon-gamma (IF- γ). It has also been reported that in rodents and humans, increased triglyceride (TG) concentrations are induced by multiple cytokines, which rapidly stimulate hepatic fatty acid synthesis[94,95] and suppress fatty acid oxidation.[96] The association between dyslipidemia and inflammation has drawn great attention in human medicine because hyperlipidemia has been associated with an increased risk of atherosclerotic cardiovascular disease (ASCVD). Moreover, the role of hyperlipidemia secondary to systemic chronic inflammation resulting in atherosclerosis remains as one of the areas of interest in human cardiovascular disease research. On the other hand, in veterinary medicine, few studies have been reported regarding dyslipidemia in dogs with inflammation, such as lymphoma,[28] parvoviral enteritis,[30] and leishmaniasis.[29] These studies revealed that dogs

with these inflammatory or infectious conditions had similar changes on lipoprotein profiles, including significantly increased plasma TG and LDL-C concentrations, and decreased HDL concentrations. The effects of infection and inflammation on TG metabolism appear to be similar in all species, while changes in cholesterol metabolism differ between species. In rodents, serum total cholesterol concentrations and hepatic cholesterol synthesis are increased. On the other hand, humans and nonhuman primates show either no change or a decrease in serum cholesterol and LDL.[72]

In addition, clinical studies and anecdotal observations suggest that hyperlipidemia is associated with hepatobiliary disease, such as diffuse vacuolar hepatopathy and gallbladder mucocele.[38] Also, primary hyperlipidemia was found to be associated with increased serum hepatic enzyme activities in clinically healthy Miniature Schnauzers.[21]

Apolipoproteins are proteins within lipoprotein particles. The main functions of apolipoproteins include providing structural components for assembly of lipoprotein particles and regulating lipoprotein metabolism.[4,5] While apolipoprotein B (apoB) is the main apolipoprotein of chylomicrones (apoB48) and LDL particles (apoB100), the main apolipoprotein of HDL is apolipoprotein AI (apoAI). ApoAI is synthesized in both the liver and the small intestine. While apoB48 is synthesized by enterocytes and secreted in chylomicrons, apoB100 is synthesized in hepatocytes and is the sole apolipoprotein in nascent VLDL.[4] Studies have shown that plasma apoB and apoAI concentrations might serve as markers for risk of cardiovascular disease and metabolic syndrome in humans.[97,98]

Chronic enteropathy (CE) is a general term used in dogs and cats that describes an enteropathy that is associated with more than 3 weeks of continuous or intermittent gastrointestinal signs, such as diarrhea and/or vomiting.[99] The term CE includes diet-responsive,

antibiotic-responsive, and steroid-responsive IBD. The pathogenesis of CE is complex and multifactorial. Chronic hepatitis is a progressive disease, which is commonly found in dogs. The majority of canine chronic hepatitis is idiopathic, but in a subset of patients, excess hepatic copper accumulation (i.e., copper-associated chronic hepatitis) can be identified.[100] The gold standard for the diagnosis of chronic hepatitis is a liver biopsy. Histopathological examination shows the presence of bridging fibrosis along with hepatocellular necrosis and inflammation.[100] Portosystemic shunts (PSSs) are vascular anomalies that connect the portal vein to the systemic circulation, bypassing the hepatic sinusoids and liver parenchyma.[101] PSSs are considered the most common hepatobiliary congenital abnormality and are most commonly seen in small/toy-breed dogs.[102,103] In dogs with PSSs, the venous blood bypasses the liver, which results in poor hepatic development, insufficient protein synthesis, altered fat and protein metabolism, and eventually liver failure. Understanding lipid metabolism and differences in lipoprotein profile patterns in dogs with gastrointestinal disease might enhance the understanding of the pathogenesis of these diseases and might even serve as a guide to tailor treatment options for some patients.

The hypotheses of this study were 1) dogs with CE or hepatic disease show an altered lipoprotein profile as assessed by CLPDP and 2) impaired synthesis of apolipoproteins in either the liver or the intestines might be a part of the mechanism of dyslipidemia in diseased subjects. Thus, the aims of this study were 1) to compare serum lipoprotein profiles as well as cholesterol, triglyceride, and apolipoprotein concentrations between dogs with CE and healthy control dogs and 2) to compare serum lipoprotein profiles between dogs with chronic hepatitis, dogs with portosystemic shunt (PSS) and healthy control dogs.

IV. 2 Materials and methods

IV. 2.1 Comparison between healthy control dogs and dogs with CE

IV. 2.1.1 Healthy control dogs

Surplus serum samples from 29 healthy dogs enrolled in another AUP approved study (IACUC 2016-0177 CA) were used. These dogs had no clinical signs or medical treatments within 6 weeks prior to study enrollment, and were defined as healthy controls based on physical examination, complete blood count, serum chemistry profile and urinalysis.

Blood samples were collected after food had been withheld for at least 12 hours. The blood samples were collected into tubes without an anticoagulant and left at room temperature for at least 40 minutes. The samples were then centrifuged for 15 minutes at 1,600 × g and 20°C, and sera were removed from the tubes. The CBC and chemistry panel were performed immediately . Leftover serum samples were stored at -80°C until lipoprotein profiling.

IV. 2.1.2 Dogs with CE

Thirty-eight dogs with CE that had continuous or intermittent gastrointestinal signs, such as diarrhea and/or vomiting for more than 3 weeks were enrolled into the study. All dogs with CE underwent a diagnostic workup, including clinicopathologic testing (i.e., complete blood count, serum chemistry profile, serum concentrations of trypsin-like immunoreactivity, cobalamin, folate, and pancreatic lipase immunoreactivity) and abdominal radiography and ultrasonography, or gastrointestinal endoscopy. Collection of serum samples was approved AUP protocol (IACUC 2015-0069 CA) after withholding food for at least 12 hours. Serum samples were shipped overnight to the GI lab with ice packs. The clinicopathologic tests were performed

on the same day or within a few days of collection. Surplus serum samples were aliquoted and stored at -80°C until further analysis.

IV. 2.2 Comparison between healthy control dogs and dogs with hepatic diseases

IV. 2.2.1 Healthy dogs

Surplus serum samples from 29 healthy dogs enrolled in another AUP approved study (IACUC 2012-101) were used. Blood collection was collected as described earlier.

IV. 2.2.2 Dogs with hepatic disease

Surplus serum samples from an unrelated study (IACUC 2014-0320) were used for this study. Serum samples from 17 dogs with chronic hepatitis, including some with copper-associated chronic hepatitis and 9 dogs with PSS were identified. Liver biopsy with histopathological examination was performed in each dog.

IV. 2.3 Assessment of lipoprotein profiles

The CLPDP was performed as described above. For statistical analysis, AUCs for nominal TRL, LDL/HDL, and HDL were determined based on the density ranges of $d < 1.0190$ g/mL, 1.0191-1.0629 g/mL, and 1.0632-1.1781 g/mL, respectively. Each AUC value was normalized by the total AUC and presented as AUC%.

IV. 2.4 Apolipoprotein measurements

Serum apoAI and apoB concentrations of 6 healthy dogs and 7 dogs with CE were measured by a commercially available nuclear magnetic resonance (NMR) assay (LabCorp, Liposcience). Linear regression of the NMR subclass signal areas against serum lipid and apolipoprotein levels measured chemically in a large study population (n=698) provided the coefficients to generate NMR-derived concentrations of apoB and apoAI. NMR-derived

concentrations of these parameters are highly correlated (r ~ 0.95) with those measured by standard methods (Description of New LP4 Algorithm, LabCorp).

IV. 2.5 Statistical analysis

The normality distribution of the data was assessed based on a Shapiro-Wilk test and visual inspection. A non-parametric test (Wilcoxon rank sum test) was performed for comparison of the measured values between diseased dogs and healthy control dogs. A chi-squared test was performed to test homogeneity on sex and breeds of the sample population. Significance was set at $p < 0.05$ for all statistical analyses. Metabolomic data analysis software, MetaboAnalyst 3.0[88] was used to generate a principal component analysis (PCA) plot in order to show the clustering/overlap among lipoprotein profiles based on AUCs of 11 subfractions in healthy control dogs, dogs with CH, and dogs with PSS. The 11 subfractions were determined by tube coordinate (every 2 mm) from the top of the tube except subfraction #1. Subfraction #1 was determined on the first peak, which corresponds to TRL.

IV. 2.6 Measurement of serum cholesterol and triglyceride concentrations

Serum cholesterol (Stanbio Cholesterol LiquiColor) and triglyceride (Stanbio Triglyceride LiquiColor) concentrations were measured using a clinical chemistry analyzer (Stanbio, Sirrus).

IV. 3 Results

IV. 3.1 Healthy dogs vs dogs with CE

IV. 3.1.1 Study population

Table 9 summarizes the signalment of the healthy dogs and the dogs with CE. Sexual status did not differ significantly between the two groups. Age and breed type (i.e., pure breed vs. mix breed) were significantly different between the two groups ($p = 0.0105$ and $p = 0.0038$, respectively).

	Healthy (n=29)	CE (n=38)	p-value
IF	4	0	
FS	14	17	
IM	1	2	
MN	10	19	$p = 0.1579$
age (yrs)	6.5 [1-12]	7 [0.5-13]	$p = 0.0105$
pure breed	15	32	
mix breed	14	6	$p = 0.0038$

Table 9 Signalment of dogs with CE and healthy control dogs.

IV. 3.1.2 Measurement of serum cholesterol and triglyceride concentrations

Serum cholesterol concentrations (Figure 14) were significantly different between dogs with CE (median [minimum-maximum]: 163 [47-432] mg/dL) and healthy control dogs (median [minimum-maximum]: 240 [129-368] mg/dL; $p = 0.0010$). In the CE group, 26 % of dogs (n=10) had serum cholesterol concentrations below the lower limit of the reference interval (reference interval: 124-335 mg/dL), while none of the healthy control dogs had cholesterol

concentrations below said lower limit of the reference interval. Serum triglyceride concentrations (Figure 15) did not differ between dogs with CE (median [minimum-maximum]: 79.5 [22-323] mg/dL) and healthy control dogs (median [minimum-maximum]: 53 [34-585] mg/dL; p = 0.2148).

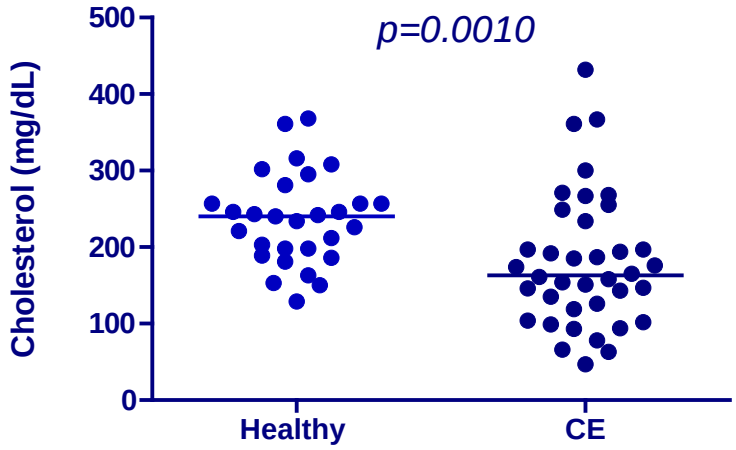

Figure 14 Comparison of serum cholesterol concentrations in dogs with CE and in healthy control dogs. The line depicts the median for each group.

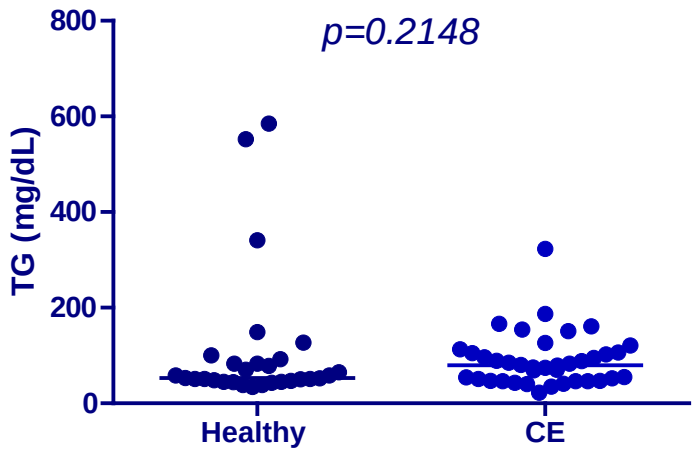

Figure 15 Comparison of serum triglyceride concentrations in dogs with CE and in healthy control dogs. The line depicts the median for each group.

IV. 3.1.3 Lipoprotein profiles

Figure 16 and 17 shows overlay graphs from healthy control dogs and dogs with CE, respectively. The AUC for TRL did not differ between two groups ($p = 0.8395$; Figure 18). The AUCs for both nominal LDL/HDL and HDL were significantly lower in dogs with CE than in healthy control dogs ($p = 0.0063$ and $p < 0.001$; Figures 19 and 20, respectively).

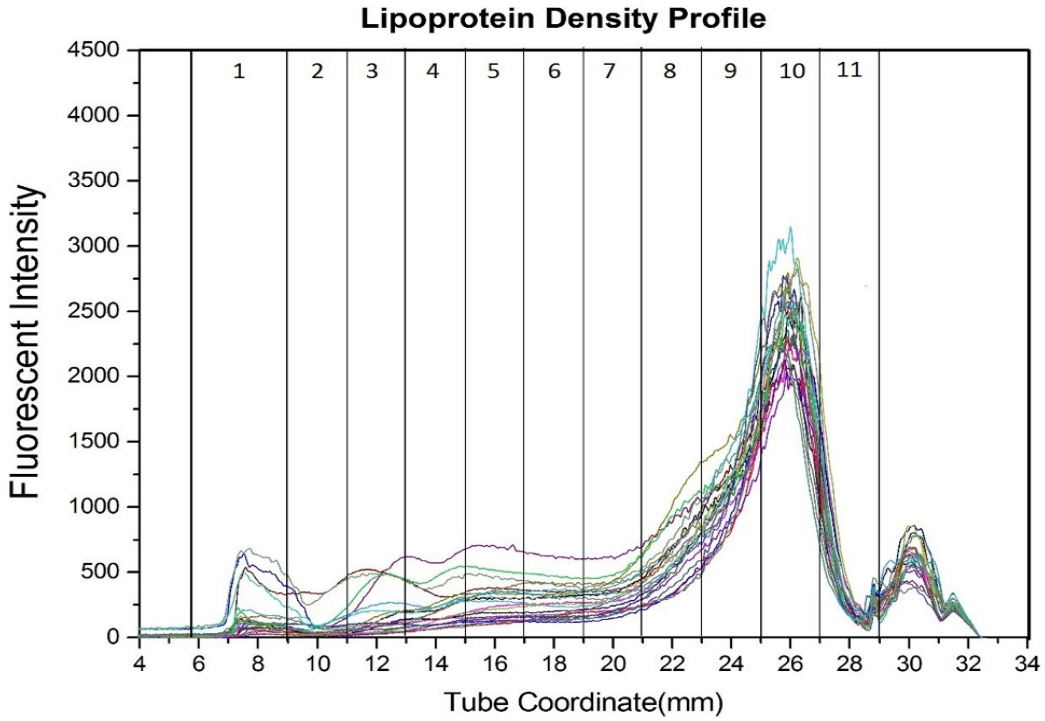

Figure 16 Overlay graphs from healthy control dogs.

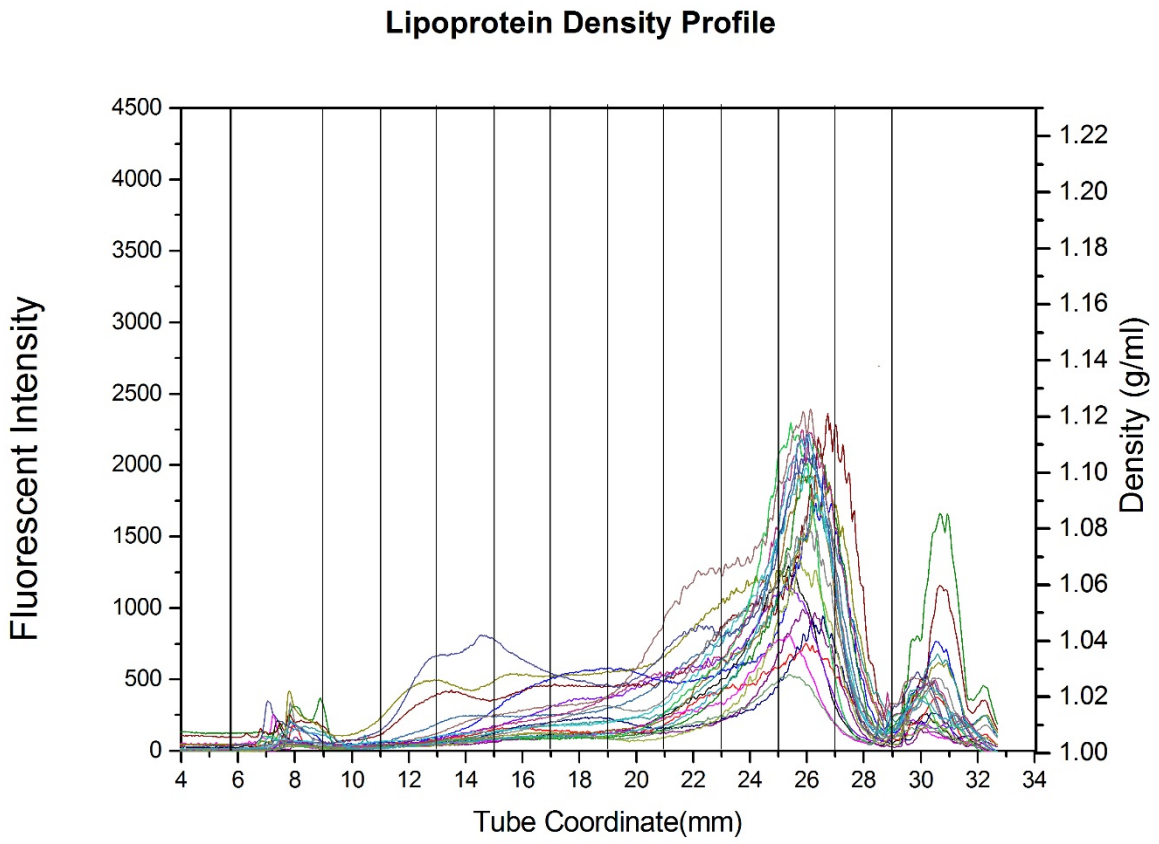

Figure 17 Overlay graphs from dogs with CE.

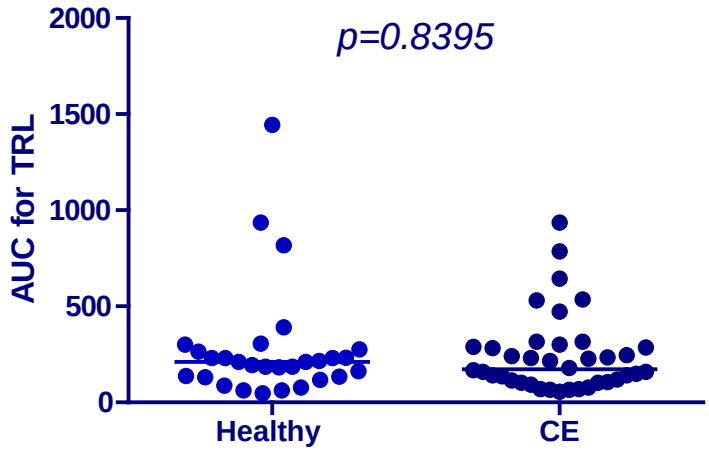

Figure 18 AUC for TRL in dogs with CE and in healthy control dogs. The line depicts the median for each group.

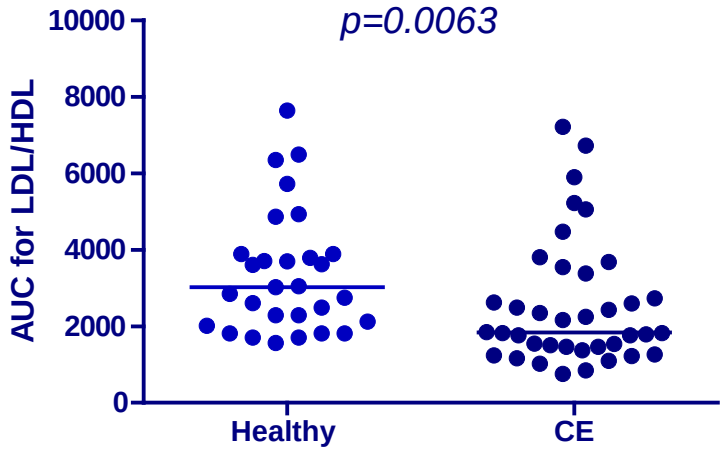

Figure 19 AUC for LDL/HDL in dogs with CE and in healthy control dogs. The line depicts the median for each group.

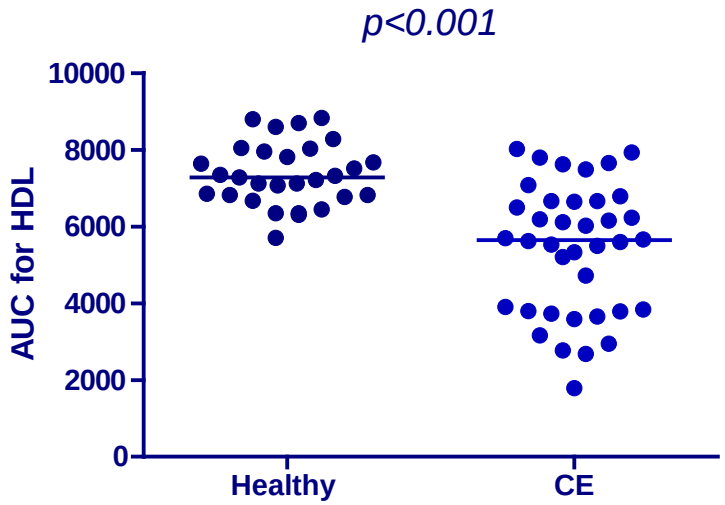

Figure 20 AUC for HDL in dogs with CE and in healthy control dogs. The line depicts the median for each group.

IV. 3.1.4 ApoB and apoAI concentrations

Serum concentrations of apoAI and apoB were measured in 6 healthy dogs and 7 dogs with CE. Serum apoB concentrations did not differ between the two groups (p = 0.9430). Serum apoAI concentrations of dogs with CE were significantly lower than that in healthy control dogs (p = 0.038; Figure 21). Figure 22 shows comparison of overlay graphs between healthy control dogs and dogs with CE that were submitted for apolipoprotein measurements.

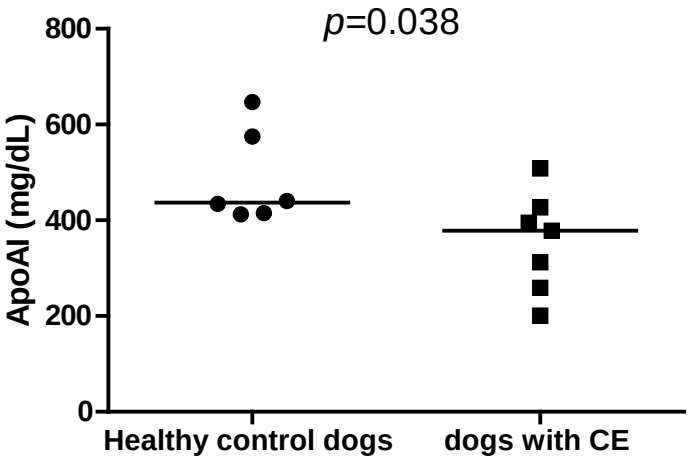

Figure 21 Serum apoAI concentrations in dogs with CE and in healthy control dogs. The line depicts the median for each group.

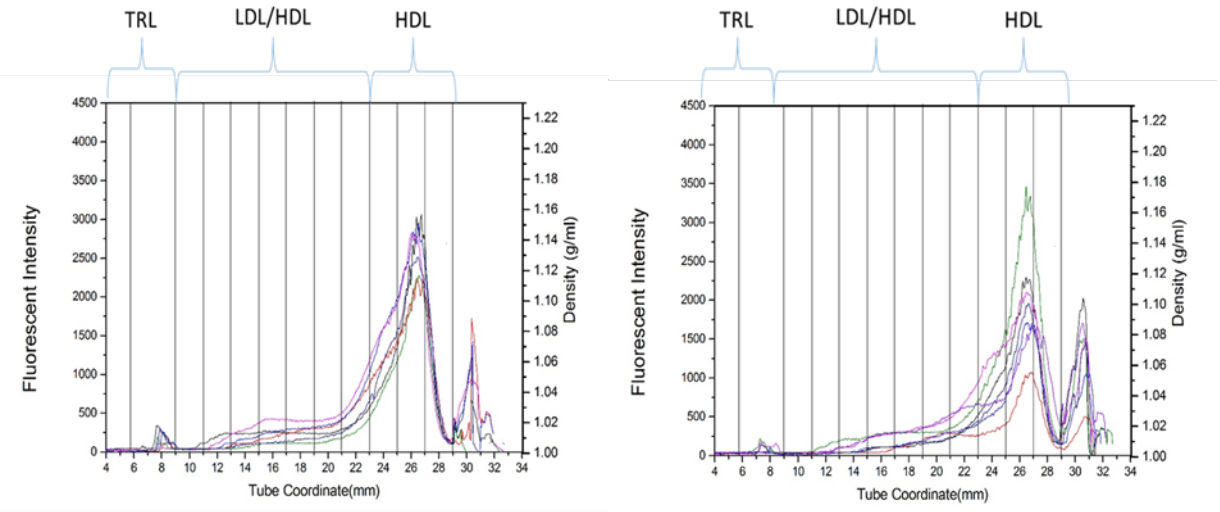

Figure 22 Overlay graphs in 6 healthy control dogs (left) and 7 dogs with CE (right).

IV. 3.2 Healthy dogs vs dogs with hepatic diseases

IV. 3.2.1 Study population

Table 10 shows the signalments of healthy control dogs and dogs with CH or PSS. Their sexual status did not differ significantly. Age and breeds distributions were significantly different among three groups ($p < 0.001$).

	Healthy (n=29)	CH (n=17)	PSS (n=9)	p-value
IF	1	2	1	
FS	10	8	1	
IM	3	2	3	
MN	15	5	4	$p = 0.3326$
age (yrs)	5 [1.5-10]	8 [3-14]	2 [0.5-5]	$p < 0.001$
pure breed	7	16	1	
mix breed	12	1	8	$p < 0.001$

Table 10 Signalments of dogs with CH or PSS and healthy control dogs.

IV. 3.2.2 Lipoprotein profiles

Figure 23, 24, and 25 show overlay graphs from healthy control dogs, dogs with CE, and dogs with PSS. Similar lipoprotein profile patterns consisting of a small peak of TRL and a high peak of HDL fractions can be seen among healthy control dogs (Figure 23). On the other hand, lipoprotein profiles in dogs with CH appeared to have higher LDL/HDL fractions compared with healthy control dogs (Figure 24). Lastly, dogs with PSS had much lower levels of LDL/HDL and HDL fractions (Figure 25). AUCs for nominal LDL/HDL were significantly different between the three groups ($p < 0.0001$). Post hoc each pair test showed significant differences between each pair (Figure 26). AUCs for nominal HDL were significantly different between the three groups ($p = 0.0005$). Dogs with PSS had significantly lower nominal HDL than the healthy control dogs or dogs with CH ($p = 0.0025$ and $p = 0.0001$, respectively; Figure 27).

AUCs% for nominal LDL/HDL were also significantly different between the three groups ($p < 0.0001$). AUC% for nominal LDL/HDL in dogs with CE was significantly higher than in dogs with PSS or healthy control dogs ($p = 0.0018$ and $p < 0.0001$, respectively; Figure 28). However, AUC% for nominal LDL/HDL between healthy control dogs and dogs with PSS did not differ ($p = 0.2572$). AUCs% for nominal HDL were significantly different between the three groups ($p < 0.0001$). AUC% for nominal HDL in dogs with CE was significantly lower than in dogs with PSS or the healthy control dogs ($p < 0.0001$ and $p = 0.0003$, respectively; Figure 29). AUCs% for nominal HDL did not differ between dogs with PSS and healthy control dogs.

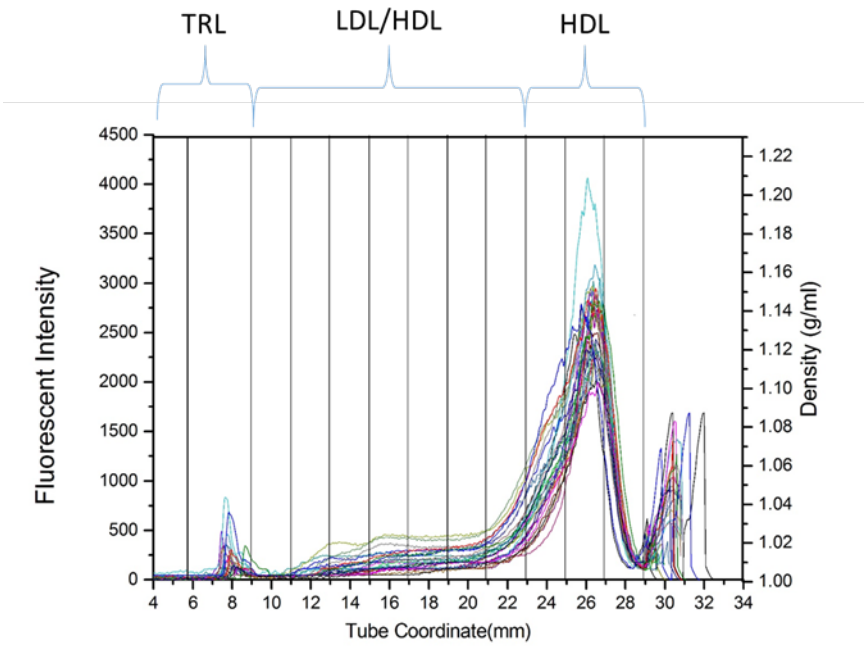

Figure 23 Overlay graphs for healthy control dogs.

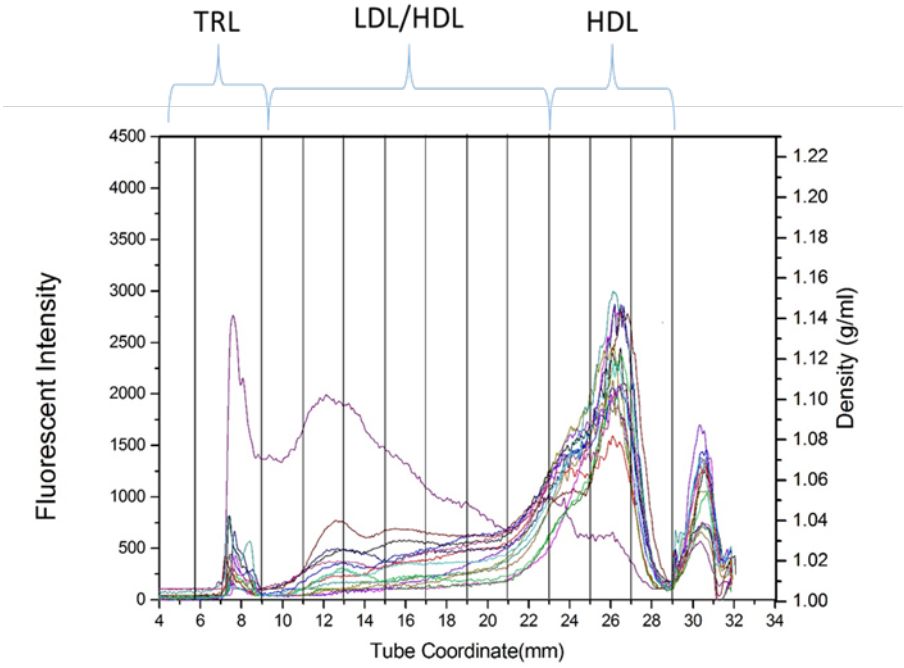

Figure 24 Overlay graphs for dogs with chronic hepatitis.

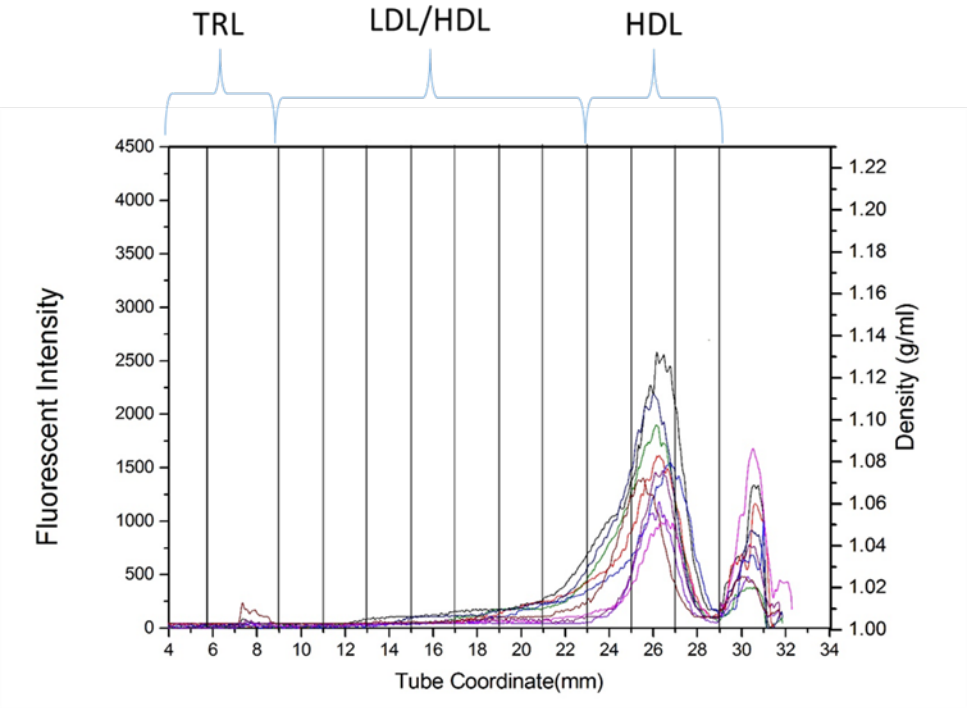

Figure 25 Overlay graphs for dogs with portosystemic shunts.

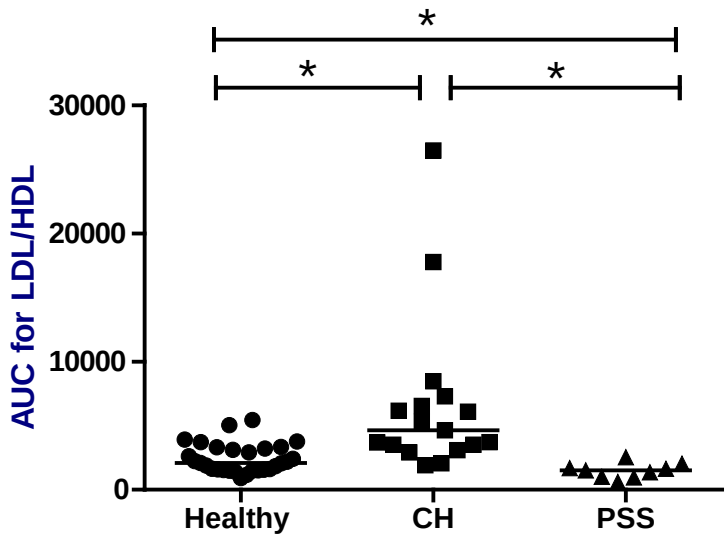

Figure 26 Comparison of AUCs for nominal LDL/HDL of healthy control dogs, dogs with CH, and dogs with PSS.

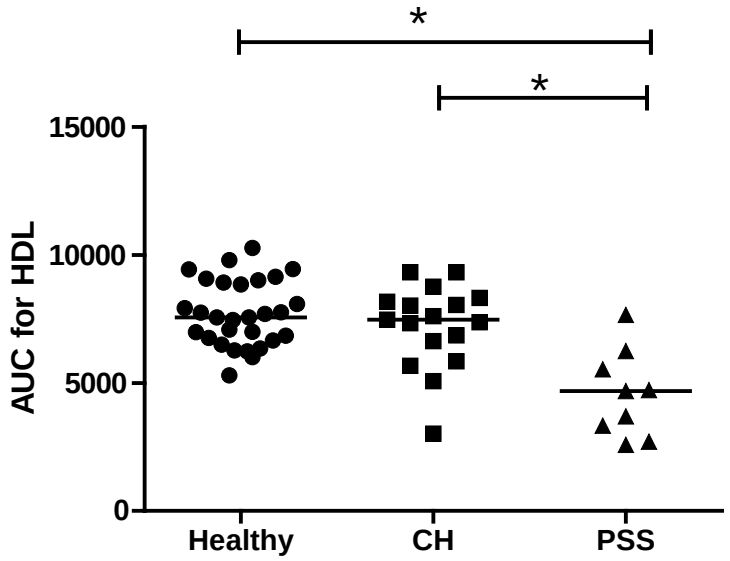

Figure 27 Comparison of AUCs for nominal HDL of healthy control dogs, dogs with CH, and dogs with PSS.

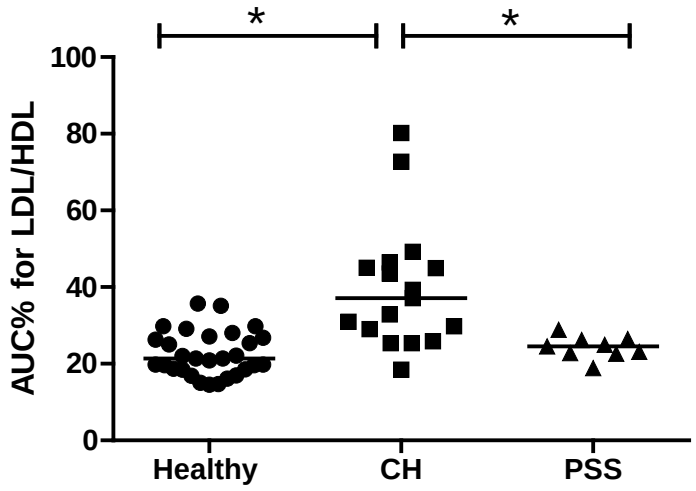

Figure 28 Comparison of AUCs% for nominal LDL/HDL of healthy control dogs, dogs with CH, and dogs with PSS.

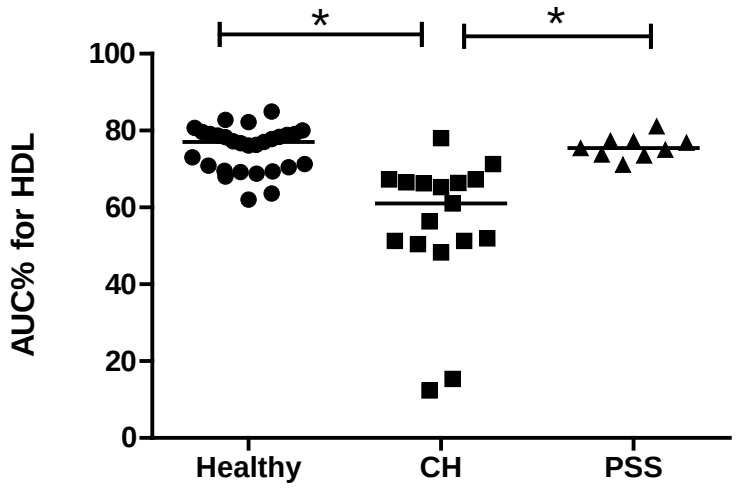

Figure 29 Comparison of AUCs% for nominal LDL/HDL of healthy control dogs, dogs with CH, and dogs with PSS.

IV. 3.2.3 PCA plot

The PCA plot (Figure 30) shows the distribution of each sample based on their similarity of lipoprotein profile composition (AUC for a total of 11 subfractions). While there is some overlap between healthy control dogs and diseased dogs, dogs with CH clustered toward the left side and dogs with PSS toward the right side.

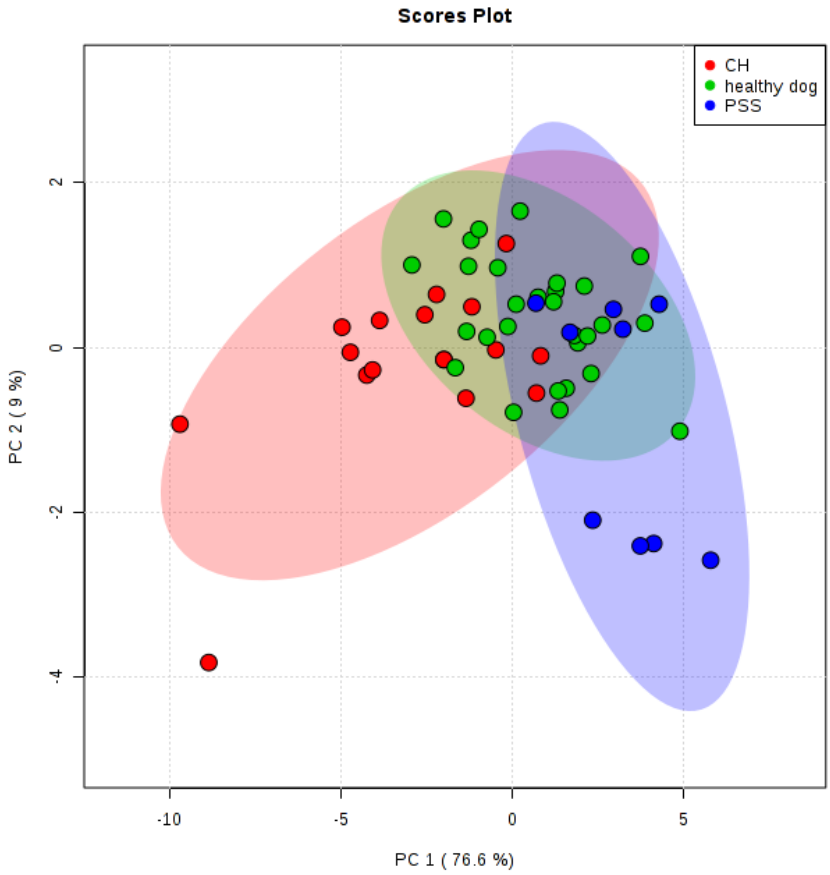

Figure 30 PCA plot of lipoprotein profiles in dogs with CH and PSS and in healthy control dogs.

IV. 4 Discussion

It has been reported that inflammation and infection cause dyslipidemia in humans and veterinary patients. Lipoproteins have important roles in physiological well-being such as maintaining cellular membrane structure and function and transporting a great amount of fat-soluble vitamins in the circulation.[91] Thus, altered lipoprotein metabolism can have significant physiological impact on affected patients. To the best of our knowledge, there are no published reports regarding lipoprotein profiles in dogs with CE, or with liver disorders, such as CH, or PSS. Assessment of lipoprotein profiles in patients may help our understanding of the

pathogenesis of these diseases, and provide novel therapeutic targets to improve treatment outcomes.

In this study, we showed that serum cholesterol concentrations were significantly decreased in dogs with CE compared with healthy control dogs, while serum triglyceride concentrations did not differ between the two groups. Dogs with CE had significantly lower LDL/HDL and HDL levels compared with healthy control dogs. In addition, NMR analysis showed that dogs with CE had significantly lower apoAI concentrations than that in healthy control dogs, while apoB concentrations did not differ between the two groups.

The altered lipoprotein profiles in dogs with CE may be due to malnutrition secondary to reduced dietary intake and/or malabsorption. In addition, several inflammatory cytokines have been thought to play roles in the alternation of lipid and lipoprotein metabolism. In fact, Buono et al. showed that dogs with CE had increased concentrations of IL-2, IL-6, and TNF-α compared with healthy control dogs (unpublished data submitted to ACVIM forum 2018). Previous studies have shown increased plasma TG concentrations in humans and mice with inflammation and infection. In this study, however, serum TG concentrations did not differ between dogs with CE and healthy control dogs. Twenty-six percent of dogs with CE in this study had serum cholesterol concentrations below the reference interval, while all healthy control dogs had cholesterol concentrations within or above the reference interval. Decreased serum cholesterol concentrations in dogs with CE were consistent with previous studies in humans. For example, children with Crohn disease are reported to have hypocholesterolemia along with reduced LDL-C.[91]

ApoAI serves as a structural component for HDL particles and constitutes 70% of HDL particles.[4] It activates LCAT and is also an anti-inflammatory molecule and an antioxidant.[104] It

was reported that apoAI mRNA expression was detected in both liver and intestine tissues in dogs but its expression in the intestine was only 15% of that in the liver.[105] Even though apoAI is mainly synthesized in the liver, decreased apoAI concentrations in dogs with CE suggest that apoAI synthesis in the intestines may be impaired because of mucosal inflammation. The reduced apoAI concentrations might have also contributed to decreased HDL levels in dogs with CE.

In addition, dogs with CH had increased nominal LDL/HDL levels resulting in increased proportions of nominal LDL/HDL and decreased proportion of nominal HDL (each AUC/total AUC). On the other hand, in dogs with PSS, the proportions of nominal LDL/HDL and HDL levels did not differ from those in healthy control dogs, while both nominal LDL/HDL and HDL levels were significantly decreased compared with healthy control dogs. These results suggest that dogs with CH may have either increased production or decreased clearance of less dense particles, such as VLDL, LDL, and less dense HDL. On the other hand, dogs with PSS have lower production of all lipoproteins due to poor hepatic development. This finding was also supported by low serum cholesterol concentrations in these dogs (data not shown).

The limitations of this study include that not all dogs with CE had histopathologal examination confirmation of mucosal inflammation. In addition, we measured serum apolipoprotein concentrations in only 6 healthy dogs and 7 dogs with CE. A bigger sample size is needed to confirm our findings.

In conclusion, we have shown that dogs with CE have lower LDL and HDL levels compared to healthy dogs. About one third of dogs with CE had serum cholesterol concentrations below the reference interval. This observed dyslipidemia might be due to inflammatory cytokines and/or impaired synthesis of apoAI in the intestines as well as

malnutrition or malassimilation. In addition, nominal LDL/HDL level was increased in dogs with CH, while both nominal LDL/HDL and HDL levels were significantly decreased in dogs with PSS. Further studies are needed to determine if addressing dyslipidemia could improve the management of dogs with gastrointestinal diseases or hepatopathies.

CHAPTER V

ALTERED LIPOPROTEIN PROFILES IN CATS WITH HEPATIC LIPIDOSIS

V.1 Introduction

Feline hepatic lipidosis (HL) is the most common hepatobiliary disease in cats and is characterized by excessive accumulation of lipid within hepatocytes, leading to intrahepatic cholestasis and severe hepatic dysfunction.[106] One study in cats with idiopathic HL showed that 34% of hepatic mass was triglycerides, compared with 1% in healthy control cats. Feline HL is classified into two forms: 1) primary idiopathic HL due to anorexia for a prolonged period of time without any identified underlying diseases (e.g., decreased food intake secondary to a stressful event or nonpalatable food) and 2) secondary HL due to an underlying disease leading to anorexia (e.g., diabetes mellitus, pancreatitis or renal failure), which is more common.[106] Although the pathophysiology of feline HL is complex and still incompletely understood, it is known that intense adipose tissue lipolysis and alterations in hepatocellular lipid metabolism during anorexia play an important role.

Previous studies have shown increased concentrations of very-low-density lipoproteins (VLDL), each lipid component (triglycerides, cholesterol and phospholipids) in VLDL and plasma nonesterified fatty acids (NEFAs) in cats with HL compared with healthy cats.[107] In addition, similarities were reported in the fatty acid composition of both liver and adipose tissues in cats with idiopathic HL.[108] These results suggest that in cats with HL, assembly of VLDL in the liver is enhanced due to excessive mobilization of fatty acids from adipose tissues to the liver. That being said, the assessment of lipoprotein profiles may improve our understanding of lipid metabolism in cats with HL, and may serve as a guide for tailored nutritional treatment. The

methodology in previous studies was a gradient ultracentrifugation (DGU) technique followed by the measurement of lipid concentrations (e.g., triglycerides or cholesterol) by enzymatic assays. The drawback of this methodology is that the DGU takes 24 hours and requires measurements of lipid components. Continuous lipoprotein density profiling (CLPDP) is a novel DGU technique, which uses a self-generating density gradient solution, bismuth sodium ethylenediaminetetraacetic acid (NaBiEDTA), and a fluorescent probe, NBD C6-ceramide.[56,74] The advantages of this technique are that it requires only one ultracentrifugation, lasting six hours, and allows visualization of a continuous distribution of lipoproteins (lipoprotein profile) and also quantification of the lipoprotein subfraction masses by calculating the area under the curve (AUC).

The CLPDP was initially developed to analyze human samples. Demacker et al. showed that cats and humans have similar lipoprotein density distributions.[109] We hypothesized that cats with HL would show altered serum lipoprotein profiles using the CLPDP compared with healthy control cats due to intensive peripheral lipolysis in adipose tissue and alterations in hepatocellular lipid metabolism. The main objective of our study was to compare lipoprotein profiles in healthy control cats and cats with HL to examine the disease associated changes in their lipid profile. A secondary objective was to examine how the CLPDP separates feline lipoproteins by examining the shapes and diameters of the lipoproteins from the resultant subfractions with a transmission electron microscope (TEM).

V.2 Materials and methods

V.2.1 Sample population

Twenty privately owned healthy cats and 23 cats with HL were enrolled into the study. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Texas A&M University (AUP2012-241) and written informed consent was obtained from all owners. Healthy cats had no clinical signs within three months prior to the study enrollment and were defined as healthy controls based on physical examination, a serum chemistry profile and urinalysis.

Cats with HL were presented with various conditions and consequent diagnostic workup was not standardized. However, diagnosis of feline HL was made based on combination of consistent clinical findings (e.g., vomiting, anorexia, weakness, or weight loss), consistent laboratory findings (e.g., elevated bilirubin, ALP or ALT), consistent abdominal ultrasound findings (e.g., diffusely hyperechoic liver) in absence of hemolysis and extrahepatic bile duct obstruction, and the diagnosis of lipid type vacuolation on cytological evaluation of the liver. The evaluation of cats for potential underlying disorders was left at the discretion of their attending veterinarians. Food was not withheld for the purpose of this study in cats with HL. However, most cats were anorexic and had not eaten for about 8 – 12 hours prior to admission per owner. The blood samples were collected into tubes without an anticoagulant and left at room temperature for at least 40 minutes. The samples were then centrifuged for 15 minutes at 1,600 × g and 20°C, and sera were removed from the tubes. Serum samples were transported with icepacks to the Gastrointestinal Laboratory at Texas A&M University. Serum samples were

kept at -80°C until lipoprotein profiling. The CLPDP for both healthy control cats and cats with HL was performed within three month of sample collection.

V.2.2 Measurement of serum cholesterol and triglyceride concentrations

Serum cholesterol (Stanbio Cholesterol LiquiColor) and triglyceride (Stanbio Triglyceride LiquiColor) concentrations were measured using a clinical chemistry analyzer (Stanbio, Sirrus).

V.2.3 Assessment of lipoprotein profiles

Lipoprotein profiles were assessed using the CLPDP technique.[110] In brief, 1,280 µL of 0.18 M NaBiEDTA density solution (Tokyo Chemical Industry) was mixed with 10 µL of serum and 10 µL of NBD C6-ceramide (Cayman Chemical Company). Then 1150 µL of the mixture was transferred into a polycarbonate centrifuge tube (Beckman Coulter). The sample tubes were centrifuged at 867,747 x g and 4^{o}C for six hours in an ultracentrifuge (Optima MAX-LP, Beckman Coulter) with a fixed angle rotor (MLA-130, Beckman Coulter). After centrifugation, the tubes were immediately imaged by a fluorescence imaging system consisting of a digital camera (Quantifire XI, Optronics) and a metal halide continuous light source (Dolan-Jenner Industries). Following ultracentrifugation, the image of each tube was converted to a density profile using a software program (OriginPro7.5, OriginLab). The tube coordinate (mm) on the X-axis of the lipoprotein profile corresponded to an actual centrifuge tube coordinate (mm). Zero mm indicated the top of the tube, and 33 mm indicated the bottom of the tube. The average intensity of fluorescence was plotted on the Y-axis to produce a lipoprotein profile. The less dense particles, such as triglyceride-rich lipoproteins (TRL), migrated near the top of the tube, whereas more dense particles, such as HDL, migrated towards the bottom of the tube. The

numbering nomenclature was created for statistical analysis. Subfraction #1 (F1) was determined based on the first peak (d < 1.0190 g/mL), which corresponds to TRL, such as chylomicrons and VLDL. Subfractions #2 (F2) to #11 (F11) were created by measuring the density of the solution collect every 2 mm (tube coordinate) from the top of the tube. Total lipoprotein intensity and fractional intensities of each region (F1-F11) were determined by measuring the area under the curve (AUC) of the entire fluorescence trace and each region (F1-F11), respectively. In addition, AUCs for nominal TRL, LDL and HDL were determined based on the density ranges of d < 1.0190 g/mL, 1.0191-1.0629 g/mL and 1.0632-1.1781 g/mL, respectively. Each AUC value was normalized by the total AUC and presented as AUC%. As a comparison, subfractions between #2 (F2) and #8 (F8) correspond to nominal LDL (1.0191-1.0629 g/mL), and subfractions between #9 (F9) and #11 (F11) correspond to nominal HDL (1.0632-1.178 g/mL).

V.2.4 Statistics

The normality distribution of the data was assessed based on a Shapiro-Wilk test and visual inspection. A non-parametric test (Wilcoxon rank sum test) and parametric test (two sample t-test) were performed where appropriate. A Chi-Square test was performed to test homogeneity on sex and breeds of the sample population. Significance was set at $P<0.05$. A metabolomic data analysis software, MetaboAnalyst 3.0[88] was used to generate a principal component analysis (PCA) plot in order to show the clustering/overlap among lipoprotein profiles based on %AUCs of 11 subfractions in healthy control cats and cats with HL. A receiver operating characteristic curve (ROC) was used to calculate sensitivity and specificity at the optimal cut-off AUC% of a lipoprotein fraction.

V.3 Results

V.3.1 Study population

Table 11 shows the breed, sexual status and age of the healthy control cats and cats with HL. Breeds and sexual status were similar. Age distribution differed significantly between healthy cats (mean± standard deviation (SD): 5.3± 2.9) and cats with HL (mean± SD: 7.8± 3.2; *P*=0.011). Underlying diseases were identified in 10 cats with HL and included pancreatitis, neoplasia, histoplasmosis, and cardiac disease; no specific underlying disease was identified in the other 13 cats.

	Healthy cats (n=20)	Cats with HL (n=23)
Breeds (n)		
DSH	13	13
DMH	5	2
DLH	2	3
Pure breeds	0	5
Sex (n)		
female spayed	9 (45%)	12 (52%)
male neutered	11	11
Age [mean± SD]	5.3± 2.9*	7.8± 3.2*

Table 11 Signalment of cats with HL and healthy control cats.
DSH: domestic short haired, DMH: domestic medium haired, DLH: domestic long haired, HL: hepatic lipidosis, **P*=0.016

V.3.2 Measurement of serum cholesterol and triglyceride concentrations

Serum cholesterol concentrations (reference interval: 73-265 g/mL) did not significantly differ between healthy cats (mean± SD: 172± 42 g/mL) and cats with HL (mean± SD: 189± 98

g/mL; *P*= 0.46). Also, serum triglyceride concentrations (reference interval: 25-133 g/mL) did not significantly differ between healthy cats (median [min-max]: 78 [32-281] g/mL) and cats with HL (median [min-max]:102 [38-1300] g/mL; *P*=0.25).

V.3.3 Lipoprotein profiles

Figure 31 and 32 display overlay lipoprotein profiles for healthy control cats and cats with HL, respectively. The Figures readily demonstrate that cats with HL have an altered lipoprotein profiles when compared with healthy control cats. The PCA plot (Figure 33) shows that lipoprotein profiles of healthy control cats cluster closely together, while the lipoprotein profiles of cats with HL are divergent from one another and encompass those of healthy control cats. Some cats with HL showed lipoprotein profiles similar to the ones observed in healthy control cats.

The AUC% for each fraction from F2 to F7 (1.0188-1.0524 g/mL) in cats with HL was significantly higher than those in healthy cats. In contrast, the AUC% for each fraction from F9 to F11 (1.0654-1.1781 g/mL) in cats with HL were significantly lower than those in healthy control cats. Density subfractions were clustered according to traditional density intervals to create nominal TRL (d < 1.0190 g/mL), LDL (1.0191-1.0629 g/mL) and HDL (1.0632-1.1781 g/mL) fraction designations.[74,109] The AUC of the nominal TRL fraction did not significantly differ between healthy control cats and cats with HL (p = 0.0796). The AUC of nominal LDL and total AUC of cats with HL were significantly greater than those of healthy control cats (P< 0.0001 and 0.0263, respectively). The AUC of nominal HDL in cats with HL was significantly lower than that in healthy control cats (p = 0.00911). When each AUC was normalized to the total AUC (AUC%), the AUC% of nominal TRL did not significantly differ between healthy

control cats and cats with HL ($p = 0.2525$). However, cats with HL had a significantly greater proportion of LDL (AUC%) than healthy control cats ($p < 0.0001$; Figure 34). Also, the AUC% of nominal HDL in cats with HL was significantly lower than healthy control cats ($p < 0.0001$; Figure 35).

Of 11 subfractions, the AUC% of F6 (1.0374-1.0438 g/mL; Figure 36) distinguished cats with HL from healthy control cats at an optimal cut-off of 7.6% with a sensitivity of 87% (95% confidence interval [CI], 73-100%), a specificity of 90% (95% CI, 80-100%) and an area under ROC curve of 93% (95% CI, 84-99%; Figure 37).

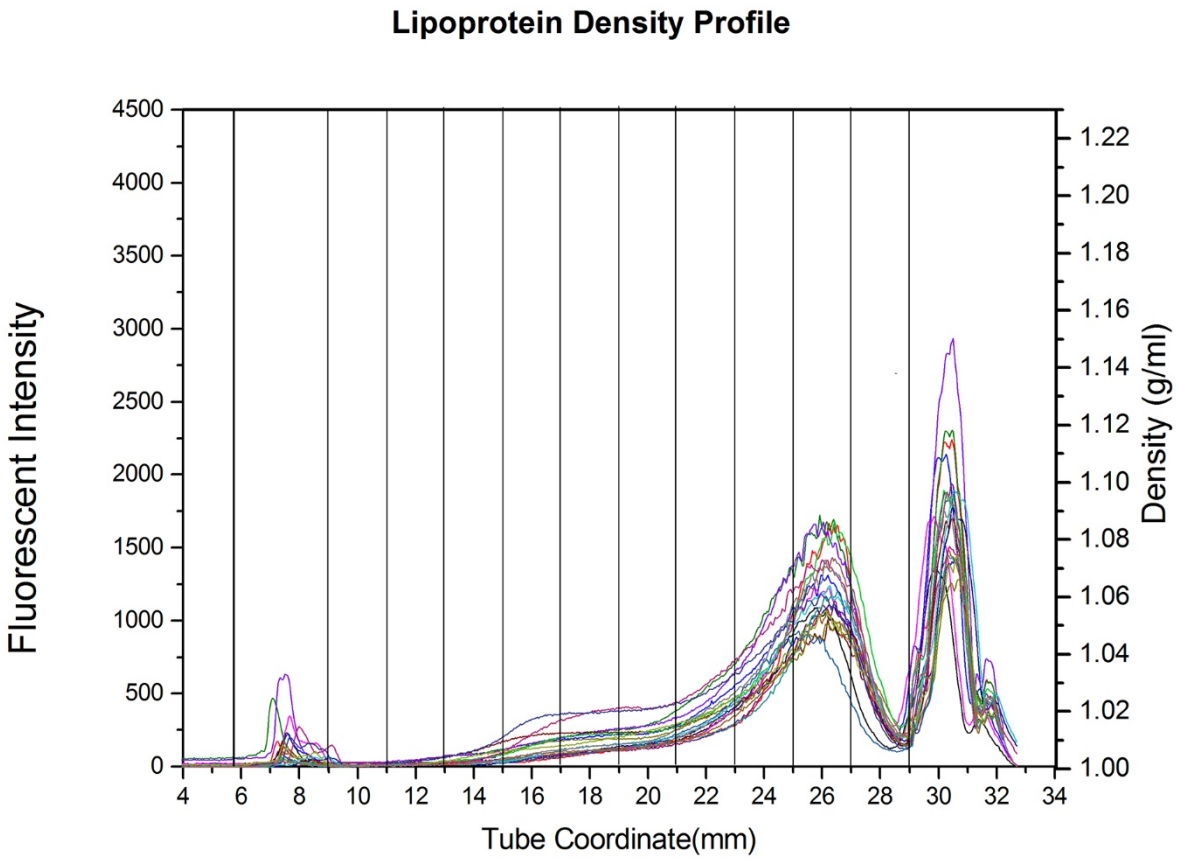

Figure 31 Overlay of lipoprotein profiles from 20 healthy control cats.

The X-axis shows the tube coordinate (mm) and the Y-axis displays the fluorescent intensity. Most cats had similar patterns characterized by a high peak at subfraction #10.

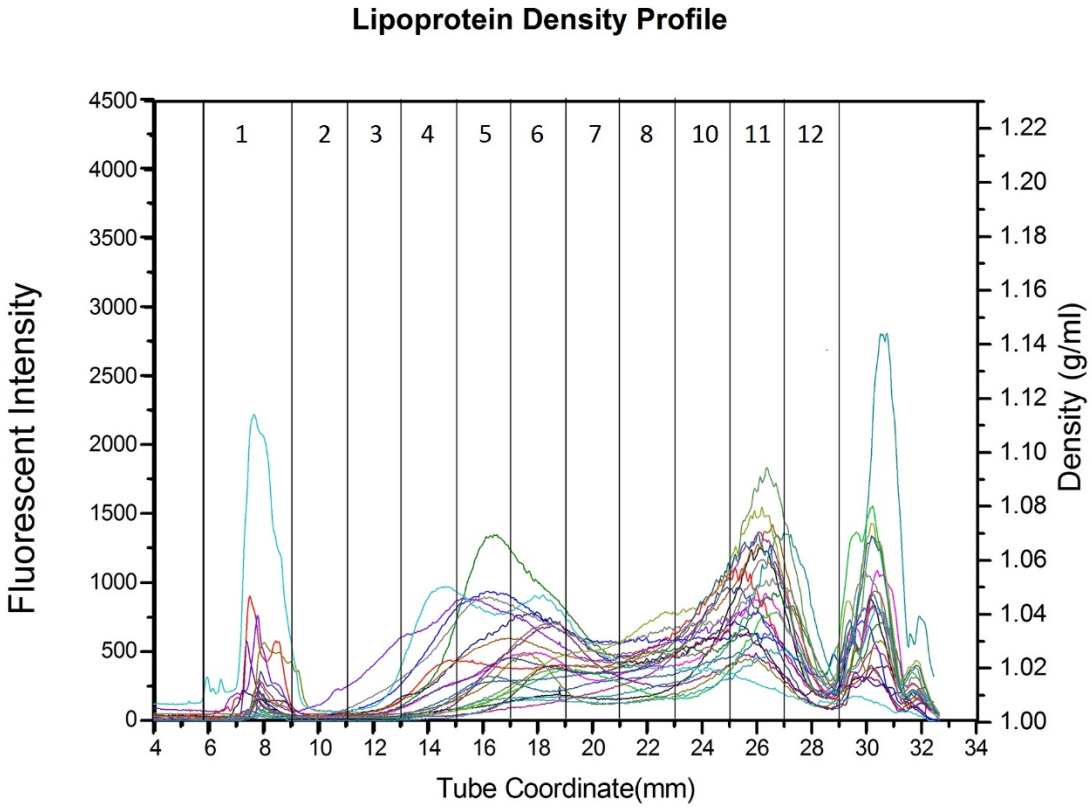

Figure 32 Overlay of lipoprotein profiles from 23 cats with hepatic lipidosis.

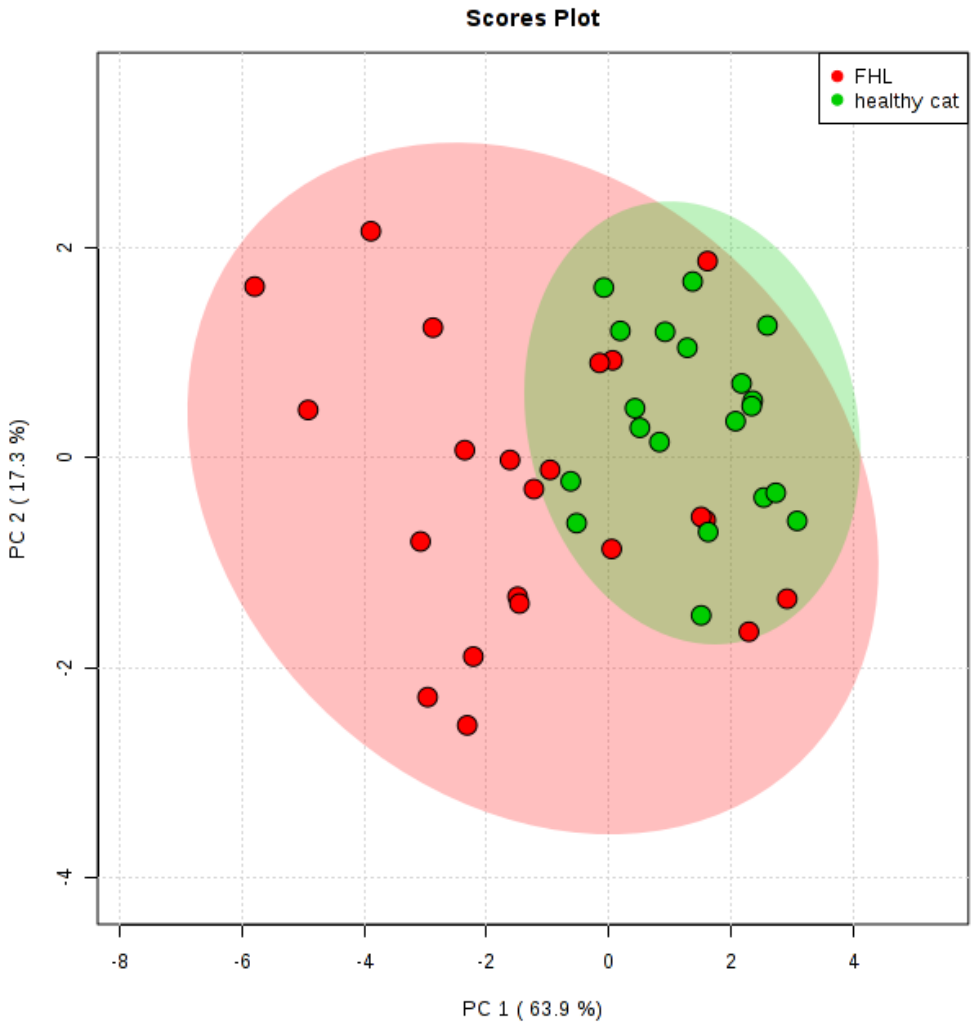

Figure 33 Principal component analysis (PCA) plot showing the relationship of lipoprotein profiles between cats with HL (red) and healthy control cats (green).

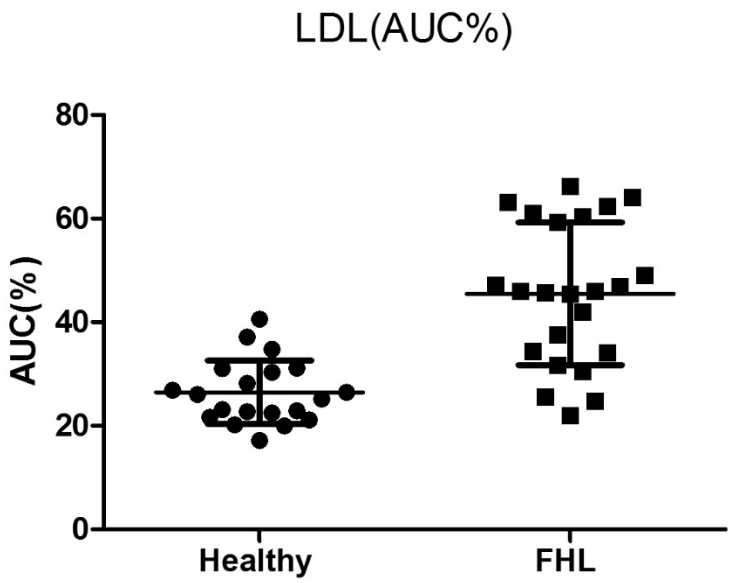

Figure 34 Comparison of area under the curve (AUC)% for low-density lipoproteins (LDL) between cats with hepatic lipidosis (FHL) and healthy control cats. *P*<0.0001.

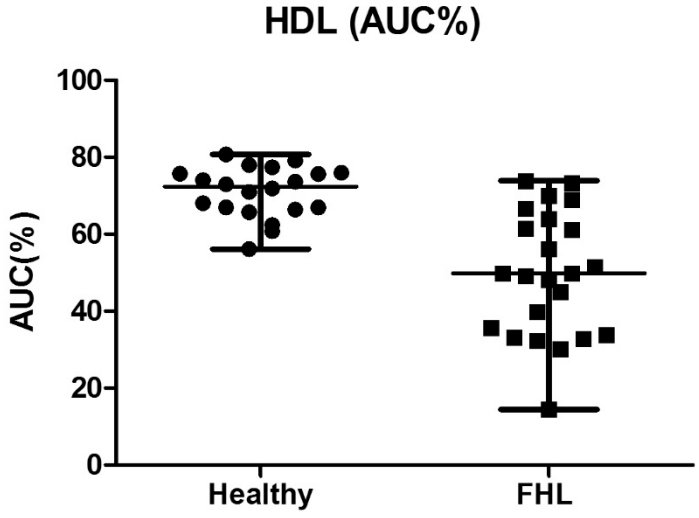

Figure 35 Comparison of the area under the curve (AUC)% for high-density lipoproteins (HDL) between cats with hepatic lipidosis (FHL) and healthy control cats. *P*<0.0001.

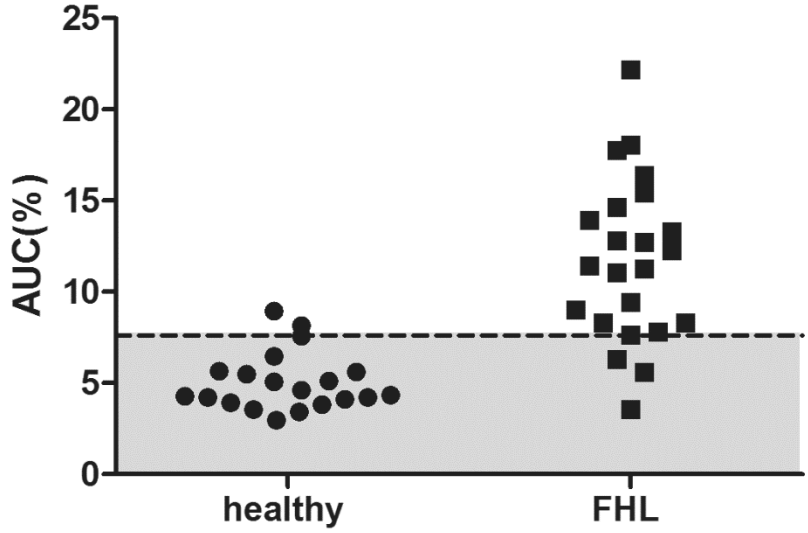

Figure 36 Comparison of fraction 6 (F6) between cats with hepatic lipidosis (FHL) and healthy control cats.

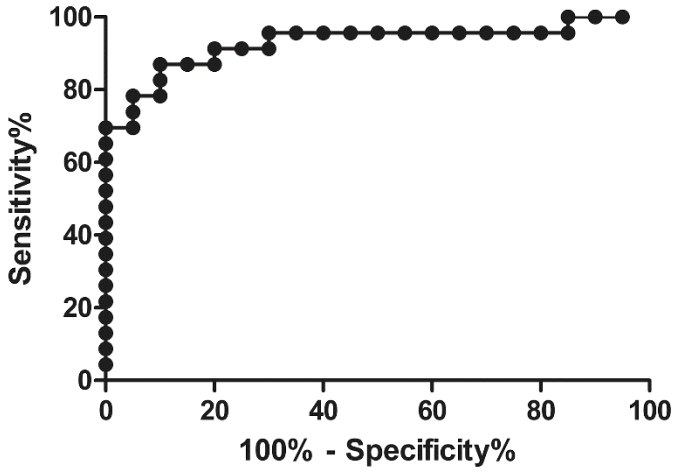

Figure 37 A receiver operating characteristic curve (ROC) of fraction 6 (F6).

V.4 Discussion

Although the pathophysiology of feline HL is not completely understood, it has been suggested that increased nonesterified fatty acids (NEFAs) mobilized from peripheral tissue due to excessive lipolysis play a pivotal role in initiating the alternation in lipoprotein metabolism in cats with HL.[111] The assessment of lipoprotein profiles in cats with HL might help better understand lipid metabolism in cats with HL. In the present study, we successfully used CLPDP to demonstrate the marked differences in lipoprotein profiles between healthy control cats and cats with HL. The advantages of the CLPDP technique include a shorter centrifugation time (6 hours) and the ability to immediately visualize lipoprotein density distributions. Moreover, the use of a fluorescent probe reduces sample volume requirements and direct imaging of density profiles obviates the need for additional chemical measurements. The CLPDP was initially developed to analyze lipoprotein density distributions (lipoprotein profile) in human plasma. Later, this technique was used to analyze lipoprotein profiles in dogs[56] as well as other species.[76,77] In the present study, electron microscopic examination showed that feline lipoproteins were spherical and the diameters of the majority of lipoproteins that fell into the LDL density range were larger than those that fell into the HDL density range as has also been reported in humans and dogs. [75,78] In addition, feline lipoproteins in this study were smaller in diameter than what has previously been reported for lipoproteins in humans and dogs.[75,78] These findings suggest that the CLPDP separates feline lipoproteins well and can be used to evaluate lipoprotein density distributions in feline serum samples.

Cats with HL in the present study had significantly higher amounts and proportions of LDL compared with healthy control cats, while TRL (VLDL) as well as serum triglyceride concentrations did not significantly differ between healthy control cats and cats with HL. It has

been proposed that various hormones associated with prolonged anorexia, such as glucagon, corticosteroids, adrenocorticotropic hormone, or catecholamines, excessively activate hormone-sensitive lipase that regulates lipolysis in adipocytes.[106] Triacylglycerol in adipocytes is hydrolyzed into three molecules of NEFA and one of glycerol. The NEFAs are bound to plasma albumin in the circulation and are taken up either by muscle cells for energy production through β-oxidation or by the liver for synthesis and/or storage of acylglycerols.[5] The glycerol is used to synthesize hepatic glucose or triglycerides. In fact, it was reported that cats with HL had increased serum NEFA and β-hydroxybutyrate concentrations.[107] The finding of an increased serum VLDL mass in some studies suggests that even though the liver is able to respond to excessive influx of NEFAs by increasing secretion of VLDL, its response may be inadequate to prevent hepatic steatosis in some cats; i.e., hepatic TG formation exceeds its secretion through the formation of VLDL. VLDL are hydrolyzed to VLDL remnants in peripheral tissues and further metabolized by hepatic lipase to become LDL.[112] However, in the present study, serum triglyceride concentrations and TRL levels did not significantly differ between healthy control cats and cats with HL. Non-elevated serum triglyceride concentrations in cats with HL was also found in a previous study.[113] These findings suggest that the liver might have a limited capacity of VLDL secretion when hepatic triglyceride accumulation was excessive in cats with HL.[113,114]

The current study also shows that cats with HL have significantly lower serum HDL compared with healthy control cats. HDL are assembled with apolipoprotein AI (apoAI).[4] Lipid-poor apoAI is secreted from the liver and intestines, and acquires phospholipids and free cholesterol from peripheral tissues and TRL to become mature HDL. This “reverse cholesterol transport” process is considered antiatherogenic in humans.[8] The mechanism of this decrease in the HDL fraction in cats with HL is still unknown. Obesity is one of the predisposing factors for

the development of feline HL[111] and obesity in humans is often associated with dyslipidemia featuring decreased HDL- cholesterol. Hepatic lipase that hydrolyzes triglyceride rich HDL shows increased activity in humans with obesity.[115] During hydrolysis of HDL mediated by hepatic lipase, lipid-poor apoA1 is released from HDL particles. Even though lipid-poor apoAI can be used to form HDL particles, it is also cleared by the kidneys.[116] Renal ApoAI loss is one of the mechanisms that could contribute to the reduced HDL that we observed in cats with HL. In addition, apoAI synthesis in the liver might be impaired due to hepatic lipidosis. Further studies to assess hepatic lipase activity and urinary apoA1 loss in cats with HL are warranted.

Measurement of pre-prandial serum cholesterol and triglyceride concentrations is currently the standard for diagnosing lipid abnormalities and assessing response to treatment.[117] In the current study, serum triglycerides and cholesterol concentrations were similar in healthy control cats and cats with HL, suggesting that both are of limited use for assessing lipid metabolism in this disease. Both density distributions (overlay graphs) and PCA plots showed a high degree of similarity for healthy control cats. In marked contrast, the profiles of cats with HL were divergent from one another and often differed dramatically from those of healthy control cats. ROC analysis showed that lipoprotein density fraction #6 (F6; d=1.037-1.043 g/mL) with a cut-off value of 7.6% had a sensitivity of 87% and a specificity of 90% for distinguishing healthy control cats and cats with HL. Given that serum triglycerides and cholesterol concentrations did not differ between healthy control cats and cats with HL in the present study, the CLPDP might be useful tool to assess lipid metabolism when HL is suspected.

There were some limitations in this study. We examined serum lipoproteins with TEM only from one healthy cat, thus this result might not represent lipoprotein sizes in other healthy cats. A larger sample size is needed to further investigate average diameters of feline

lipoproteins. The other limitations of this study include the fact that we were unable to collect follow-up samples from the cats with HL. Sequential sampling may provide insight about how lipid metabolism changes in response to treatment, and would help to investigate whether the lipoprotein profiling result might serve as a prognostic marker for cats with HL.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Lipids are defined primarily based on their solubility, rather than their chemical structures.[5] According to this definition, lipids are insoluble in water but soluble in non-aqueous solvents. Lipids, which include triglycerides, cholesterol, fatty acids, and phospholipids, are necessary to maintain homeostasis. For example, cholesterol is the essential precursor for bile acids, corticoid steroids, sex hormones, and vitamin D-derived hormones. Phospholipids are the backbone component of cell membranes. Because lipids are insoluble in an aqueous environment, they need to be packaged into lipoprotein particles before they can travel in the blood stream or cross cell membranes. Lipoproteins are conglomerates of lipids and proteins. In humans, lipoproteins are commonly categorized into 5 classes based on their densities: chylomicrons, very low density lipoproteins (VLDLs), intermediate-density lipoproteins (IDLs), low density lipoproteins (LDLs), and high density lipoproteins (HDLs). Chylomicrons are the largest particles and are considered exogenous lipoproteins. The main function of chylomicrons is to transport dietary TG from the small intestine to adipose tissue and skeletal/cardiac muscle as well as to transport dietary cholesterol to the liver.[4] Another important function of chylomicrons is to carry fat-soluble vitamins, such as vitamin A, D, E and K.[5] The chylomicron remnants are taken up by the liver and some vitamins are stored in the adipocytes of the liver. The other classes of lipoproteins are considered endogenous lipoproteins.[1] VLDLs are secreted from the liver in order to export endogenously synthesized triglycerides and to deliver triglycerides to adipose and muscle tissue. VLDLs lose triacylglycerol and apolipoproteins, and become smaller and denser particles, i.e., LDLs. LDLs mainly transport cholesterol to peripheral tissues and the liver. HDLs serve to remove cholesterol from peripheral cells and macrophages,

and transport the cholesterol to the liver. This process of “reverse cholesterol transport” is considered antiatherogenic in humans.

Atherosclerotic cardiovascular disease (ASCVD) affects more than one-third of the adult human population and accounts for 35% of all US deaths.[7] The atherosclerotic process is complex but appears to be initiated by the retention of LDL particles. Epidemiological studies have shown that increased plasma LDL-C and decreased plasma HDL-C concentrations are highly associated with an increased risk of ASCVD.[118] The obvious importance of ASCVD in the human population has led to extensive research on the subject. Thus, there is a wealth of data on lipoprotein metabolism in humans and laboratory animals. Unlike in human medicine, hyperlipidemia has been considered a relatively benign condition in dogs and cats. In veterinary medicine, measurement of pre-prandial serum cholesterol and triglyceride concentrations is currently the standard for diagnosing lipid abnormalities. The clinical usefulness of measurement of the plasma cholesterol concentration is limited in dogs and cats, mainly because abnormalities in plasma cholesterol concentrations in companion animals are not associated with the same complications seen in humans. On the other hand, hypertriglyceridemia has been reported to be a risk factor for pancreatitis,[35] hepatobiliary disease,[36] atherosclerosis,[37] systemic xanthomatosis,[18] and ocular diseases in dogs and cats.[38,39] Previous studies have described lipoprotein profiles in dogs with Leishmaniasis,[29] obesity,[24,25,119] lymphoma,[28] dominance aggression,[27] parvoviral enteritis,[30] and in cats with hepatic lipidosis.[113,120] Lipoprotein profiles in these studies included serum/plasma concentrations of TG, cholesterol, fatty acids, VLDL-C, LDL-C, and HDL- C. The most common technique for the assessment of lipoprotein profiles in these studies is the estimation of cholesterol concentrations in LDL and HDL particles by chemical precipitation of apoB-containing particles (i.e., LDLs) and the Friedewald calculation method.[49] However,

lipoproteins are highly heterogeneous particles, and cholesterol concentrations within the lipoprotein particles do not necessarily reflect their biological properties.[73] Therefore, it is more meaningful to evaluate the entire lipoprotein distribution rather than cholesterol concentrations within lipoprotein particles.[73,74] In addition, it has been reported that the precipitation technique is not suitable for canine lipoprotein analysis because of co-precipitation with ApoA-containing particles (HDLs).[79] Some studies used an ultracentrifugation technique and/or liquid-chromatography to separate lipoprotein fractions and measured the lipid content, such as TG, cholesterol, phospholipid, within these fractions by enzymatic assays. However, these techniques are often very time-consuming because they require sequential centrifugation steps and are followed by enzymatic assays.

Continuous lipoprotein density profiling (CLPDP) is a novel DGU technique, which uses a self-generating density gradient solution, bismuth sodium ethylenediaminetetraacetic acid (NaBiEDTA), and a fluorescent probe, NBD C6-ceramide.[56] The advantages of this technique are that it requires only one ultracentrifugation step, lasting 6 hours, and it allows the investigator to visualize a continuous distribution of lipoproteins (lipoprotein profile) and to estimate the concentrations of lipoprotein subfractions by calculating the area under the curve.

The hypotheses of this study were that 1) CLPDP would be a useful tool for the assessment of lipoprotein profiles in dogs and cats, and that 2) dogs and cats with gastrointestinal disease or hepatic disease have altered lipoprotein profiles as measured by CLPDP when compared to healthy controls.

Prior to assessing lipoprotein profiles in serum samples, we conducted a partial assay validation for use of the methodology with canine serum samples; we also conducted an evaluation of the effect of freeze-thaw cycles on canine serum samples, and short- and long- term

stability studies on canine serum samples kept under different storage conditions. Our partial assay validation shows that the CLPDP was repeatable and reproducible. The effect of up to three freeze-thaw cycles after storing at -80°C was acceptable for the density ranges of nominal LDL/HDL and HDL. However, storing at -80°C may not be suitable for evaluating the density range of TRL and therefore, fresh samples may need to be used. We also found that lipoproteins in canine serum that are in the range of nominal LDL/HDL and HDL, are stable for up to 1 month at 4°C and -20°C as well as for up to 12 months when stored at -80°C. It is important to note that the lipoprotein classification used in humans does not accurately represent the canine and feline lipoprotein distribution because lipoprotein metabolism varies among species. Thus, in order to investigate how the CLPDP separates canine and feline lipoproteins, we examined canine and feline lipoprotein particles in the density ranges of nominal LDL/HDL and HDL with electron microscopy. Negative-staining electron microscopy showed both canine and feline lipoproteins as spherical particles in both density ranges. In canine serum samples, there was an overlap of particle diameters between the two density ranges with the density range of LDL showing larger particles, which is one of characteristics of LDL particles. On the other hand, in feline serum, the majority of lipoproteins within the nominal LDL/HDL range were larger than those within the nominal HDL range, and there was much less overlap of particle diameters between the two density ranges compared with what has been observed in canine serum. In addition, overall feline lipoproteins were smaller in diameter than canine lipoproteins. These findings suggest that the CLPDP can be used to evaluate canine and feline lipoprotein profiles. However, it is important to note that there is a distinct overlap between the LDL and HDL density ranges that have been established for used humans, though less so in feline than in canine lipoproteins.

Our study showed that lipoprotein profiles in dogs with EPI had significantly lower LDL and HDL levels compared to healthy control dogs. Moreover, dogs with EPI that had not yet been treated with enzyme supplementation (EPI-NT) had significantly lower lipoprotein levels than dogs with EPI that were already being treated with enzyme supplementation (EPI-T). These findings can be explained by the pathogenesis of EPI: the lack of digestive enzymes in the small intestinal lumen leads to insufficient absorption of dietary TG and cholesterol, which in turn may lead to dogs with untreated EPI having insufficient VLDL and LDL. In addition, they may not have enough free cholesterol in peripheral tissues and/or TRL to synthesize optimal numbers of HDL particles. A previous study showed that dogs with experimentally induced EPI (n=8) had elevated serum alanine aminotransferase (ALT), aspartate aminotransferase, and alkaline phosphatase activities.[121] In addition, histopathological examination of liver specimens revealed mild to moderate hepatic lipidosis in all these dogs. Hepatic lipidosis in dogs with EPI may be the result of maldigestion, malnutrition, and decreased synthesis of apolipoproteins in the liver. In the present study, serum ALT was mildly elevated in dogs with EPI. Whether the dogs with EPI in our study had hepatic lipidosis is unknown, but hepatic lipidosis might be attributed to altered lipoprotein metabolism in dogs with this disorder Lipoprotein profiles can be used to assess lipid metabolism in dogs with EPI. For example, the lipoprotein profile may be a useful tool for evaluating dyslipidemia as well as monitoring the efficacy of enzyme supplementation in dogs with EPI.

We also investigated lipoprotein profiles in healthy dogs and dogs with chronic enteropathy (CE). Our results showed that AUCs for total lipoproteins, nominal LDL/HDL, and nominal HDL in dogs with CE were significantly lower than those in healthy control dogs. Serum cholesterol concentrations in dogs with CE were significantly lower than controls.

Moreover, 26 % of dogs with CE (n=10) had serum cholesterol concentrations below the lower limit of the reference interval (RI: 124-335 mg/dL), while all the healthy control dogs had serum cholesterol concentrations within or even above the RI. In addition, serum ApoAI concentrations in dogs with CE were significantly lower than in healthy control dogs, while serum ApoB concentrations did not differ between the two groups. ApoAI serves as a structural component for HDL particles and constitutes 70% of HDL particles.[4] It has been reported that ApoAI mRNA expression can be detected in both hepatic and intestinal tissues in dogs but its expression in the intestine was only 15% of that in the liver.[105] Even though ApoAI is mainly synthesized in the liver, decreased ApoAI concentrations in dogs with CE suggests that ApoAI synthesis in the intestines may be impaired because of mucosal inflammation. The reduced ApoAI concentrations might also be contributing to decreased HDL levels in dogs with CE. Altered lipoprotein profiles in dogs with CE in this study may be due to reduced lipoprotein lipase activity, decreased absorption of ApoAI from the small intestine, decreased synthesis of ApoAI in the small intestine and the liver, and malabsorption of dietary fat from the small intestines. Our results for HDL level and serum cholesterol concentrations were consistent with the previous studies in human patients with IBD. However, human patients with IBD had significantly higher LDL-cholesterol concentrations.

Assessment of lipoprotein profiles in dogs and cats with liver diseases was also performed. Dogs with chronic hepatitis (CH) had increased levels of nominal LDL/HDL when compared to healthy control dogs, whereas as nominal HDL did not differ. These results suggest that dogs with CH may have either an increased synthesis or decreased clearance of less dense particles, such as VLDL, LDL, and less dense HDL. On the other hand, dogs with PSS had significantly decreased levels of both nominal LDL/HDL and HDL when compared to dogs with

CH or healthy control dogs. These results indicate that dogs with PSS produce less of every type of lipoprotein due to poor hepatic development. This finding was also supported by low serum cholesterol concentrations in these dogs.

Our study also showed that cats with HL had elevated nominal LDL/HDL levels and deceased HDL levels compared to healthy control cats. Importantly, even though lipid metabolism in cats with HL is presumed to be altered as part of the disease process, our study showed that serum cholesterol and triglyceride concentrations are similar between cats with HL and healthy control cats. On the other hand, ROC analysis showed that lipoprotein density fraction #6 (F6; d=1.0374-1.0438 g/mL) with a cut-off value of 7.6% had a sensitivity of 87% and a specificity of 90% for distinguishing cats with HL from healthy control cats. For a better comparison of the clinical usefulness of serum TG and cholesterol concentration and the F6, 25% of cats with HL had serum cholesterol concentrations above the upper limit of the reference interval, while this value was only 5% for healthy control cats. Also, 33% of cats with HL had serum triglyceride concentrations above the upper limit of the reference interval, whereas this analyte was increased in only 15% in healthy control cats. On the other hand, 85% of cats with HL had the F6 above the cut-off value of 7.6% compared with only 10% of the control cats. These results support the clinical usefulness of the lipoprotein profiling in cats with HL. A definitive diagnosis of feline hepatic lipidosis requires cytological and histological confirmation of diffuse hepatocellular triglyceride vacuolation. However, cats with severe HL are often substantially compromised and may be at risk of anesthetic complications and/or a bleeding diathesis, which complicates acquisition of hepatic fine-needle aspirates and biopsies of the liver. In addition, because of the added financial burden, clinicians may be hesitant to perform ultrasound-guided needle aspiration. In these cases, a lipoprotein profile might help to support a clinical suspicion of HL when paired with other blood

tests, such as CBC, measurement of liver enzyme activities (e.g., ALP, ALT and GGT), electrolytes, and coagulation tests, especially because lipoprotein profiling is cheaper and less invasive compared to ultrasound-guided needle aspiration. In our study, a cat with HL who responded to supportive care showed a shift in the altered lipoprotein profile to a normal pattern within 2 weeks. "Normal pattern" was defined by the lipoprotein profiles observed in healthy control cats. The clinical signs of this cat correlated well with the improvement of its lipoprotein profile, suggesting that this could be used as a tool for monitoring the lipid metabolism in cats with HL. Further studies with a larger sample size are needed to confirm whether normalization of the lipoprotein profile is consistently associated with an improvement in clinical signs and biochemical parameters.

Given that lipids are crucial for maintaining homeostasis, dyslipidemia might play a role in initiation of complications and poor response to treatment, which may negatively affect quality of life in dogs and cats with gastrointestinal and hepatic diseases. For example, it has been suggested that HDLs possess anti-inflammatory properties and may play a role in innate immunity by regulating the inflammatory response in patients with gram-negative sepsis.[122] The mechanism of the protective effect of HDLs is complex and not completely understood. However, *in vivo* and *in vitro* studies have shown that LPS binds to and is neutralized by lipid emulsions, chylomicrons, VLDLs, LDLs, HDLs, ApoAI and ApoE, with some authors suggesting that HDLs show the most potent binding.[95,122,123] In other words, dyslipidemia in patients with an infection can be viewed as a physiological protective response to pathogens. In fact, a previous study in human sepsis patients showed that non-survivors had significantly lower concentrations of cholesterol, TG, HDL, LDL, and ApoAI compared to survivors.[124] In addition, their results indicated that decreased TG concentrations are associated with a higher mortality in

patients with sepsis. As another example, impaired fat absorption from the small intestine manifested by decreased serum chylomicron concentrations can lead to vitamin deficiencies. Impaired fat absorption from the small intestine might be a result of decreased secretion of bile acids or damaged intestinal epithelial cells, which can be caused by inflammation or infection.

In summary, assessment of lipoprotein profiles in patients with gastrointestinal or liver disease may improve our understanding of certain aspects of the pathogenesis of these diseases, and may also provide a basis for novel therapeutic approaches. In addition, further studies are needed to investigate whether identification and management of dyslipidemia may improve outcome and quality of life in dogs and cats with gastrointestinal disease or liver disease.

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